EFFECTS OF AGE AND DIET ON GLUCOSE AND INSULIN DYNAMICS IN THE HORSE

By

Julie Lynn Rapson

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Large Animal Clinical Sciences – Master of Science

2015

ABSTRACT

EFFECTS OF AGE AND DIET ON GLUCOSE AND INSULIN DYNAMICS IN THE HORSE

By

Julie Lynn Rapson

Insulin resistance (IR) is a key metabolic disturbance in horses that develop obesity-associated laminitis. In addition to obesity, age and diet affect tissue sensitivity to insulin but these factors have received limited investigation in horses. This study tested the hypothesis that glucose and insulin responses to a sweet feed (SF, high CHO) meal would be greater in aged horses, as compared to adult horses, as well as in horses adapted to a forage-only diet. Three diets, grass hay (G), grass hay plus sweet feed (starch and sugar-rich, SS), and grass hay plus a fat and fiber feed (FF), were fed to 17 healthy mares, 8 adult (5-12 yr) and 9 aged (>19 yr), for a 6-week adaptation period in a randomized design. Minimal model parameters during a frequently sampled intravenous glucose tolerance test (FSIGTT) and glucose and insulin responses to a standardized SF meal were determined after 30 and 42 days on each diet, respectively. Data were analyzed by repeated measures ANOVA. The acute insulin response to glucose (AIRg) was greater and tissue insulin sensitivity was lower in aged horses, regardless of diet. No differences in glucose responses to the SF meal were detected between age groups for any of the diets; however, both peak glucose concentration and AUC-G were lower after adaptation to the SS diet. In contrast, peak insulin concentration and AUC-I were greater in aged horses than adult horses on all diets but no differences were found between diets within age groups. As hypothesized, the insulin response, but not the glycemic response, to a sweet feed meal was greater in aged horses, regardless of background diet. Further, the glycemic response was greatest after adaptation to a forage-only diet in aged horses only.

ACKNOWLEDGMENTS

I would like to thank my committee members Dr. Schott, Dr. Geor, and Dr. McCutcheon for their continued guidance throughout this process.

I would also like to thank my family and friends for their support. Lastly, I would like to recognize all of the horses that were a part of this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

This thesis contains three chapters. The first chapter is a literature review that describes reduced tissue sensitivity to insulin called insulin resistance. Insulin plays and important role in carbohydrate and lipid metabolism. A multitude of different factors that can affect insulin resistance are discussed in humans as well as horses. Insulin resistance is evaluated by both non-specific and specific methods with the two standard techniques used in a research setting being the euglycemic-hyperinsulinemic clamp and minimal model analysis of insulin and glucose dynamics during a frequently sampled intravenous glucose tolerance test. There are limitations to both of these methods which have lead to the development of less invasive techniques for measuring insulin sensitivity such as the meal response.

The second chapter describes the experimental protocol and results of the minimal model analysis using frequently sampled insulin glucose tolerance test and a meal response challenge in both adult and aged horses.

In the third chapter, conclusions are made based on previous research. Additionally, possible underlying mechanisms for age and diet variations in tissue sensitivity to insulin in the horse are discussed. Lastly, practical implications from introducing a high sugar and starch concentrate to horses with metabolic disease are considered.

CHAPTER 1

LITERATURE REVIEW

Overview

Insulin resistance is defined as a decreased response of peripheral tissues to a normal concentration of insulin. As a result, a greater amount of insulin is required to attain a biological effect. Insulin resistance is a component of many metabolic conditions such as type II diabetes and is associated with other health problems such as obesity in both humans and horses. Specific to the horse, hyperinsulinemia and insulin resistance predispose horses to laminitis which is a painful, life threatening disease of the hooves [19]. The term Metabolic Syndrome has been used to describe a combination of different medical disorders that increase the risk of developing diseases such as diabetes and hypertension in humans. Recently, the term Equine Metabolic Syndrome (EMS) has been coined to describe a clinical syndrome of obesity, insulin resistance, and predisposition to laminitis in horses [35].

There are many factors that can influence insulin sensitivity in both humans and horses. Two of these factors are age and diet. In both humans and horses, insulin sensitivity decreases with age leading to an increased prevalence of insulin resistance [80]. The appropriate diet for animals and humans that are insulin resistant is a subject of extensive research today. Both carbohydrate restriction and increased protein consumption increase insulin secretion in humans [113]. In equids, there is a great deal of debate as to what diet is most appropriate for an aged horse with decreased insulin sensitivity, as well as for an animal diagnosed with EMS. There are low starch diets and senior feeds marketed for the aged equid but limited research has been conducted to determine whether these are the most appropriate diet for the insulin resistant or

EMS horse. Therefore, the scope of this study was to look at the effects of age and diet on glucose and insulin responses of a cohort of healthy, non-obese mares.

Insulin

Insulin is the crucial hormone involved in regulation of carbohydrate and fat metabolism. Storage of nutrients is the major function of insulin. Insulin is a secretory protein consisting of two peptide chains: an A chain consisting of 21 amino acids and a B chain with 30 amino acids. These two chains are joined by two disulfide bridges giving mature insulin a total of 51 amino acids. The half-life of endogenous insulin in circulation is 10 minutes [13].

Insulin is produced in the endocrine portion of the pancreas, the islets of Langerhans. The islets of Langerhans contain different types of secretory cells including α cells, β cells, δ cells, and F cells. Beta cells are located within the central core of the islets and produce insulin, proinsulin, amylin, and C-peptide. Insulin is encoded by a gene located on the short arm of chromosome 11. Transcription of this gene produces mRNA that encodes preproinsulin. Shortly after it is synthesized, the signal sequence is cleaved from the N-terminus of preproinsulin resulting in proinsulin. Proinsulin consists of three domains: A, B, and C. Proinsulin is packaged into clathrin-coated secretory granules within the Golgi apparatus. While in the Golgi apparatus, proteases cleave proinsulin at two sites resulting in a 31-amino acid connecting or C-peptide and insulin, consisting of chains A and B linked by disulfide bonds. Maturation of the secretory granule is completed with the loss of the clathrin coating. A mature secretory vesicle released into the portal circulation contains insulin and C-peptide in equal quantities in addition to a smaller amount of proinsulin [9]. Release of insulin is biphasic with the acute phase representing release of preformed insulin in secretory granules and the later phase associated with *de novo* synthesis of insulin [13].

Once released into the portal blood, insulin travels to the liver where over half is bound, exerts its action, and is removed immediately from circulation. The remaining insulin is available to act on other target tissues within the body. Once at a target tissue, insulin binds to specific receptors on the plasma membrane. The insulin receptor is a heterotetramer consisting of two identical, extracellular α subunits and two β subunits that span the width of the plasma membrane. The β subunits have an extracellular domain that must be glycosylated in order for insulin binding to occur. The intracellular domain of the β subunit is coupled to tyrosine kinase, required for activation of downstream signaling pathways [9].

Insulin binds to the α subunit of the insulin receptor. This binding signals the β subunit to autophosphorylate resulting in activation of the β subunit. The activated β subunit will recruit proteins as well as activate additional substrates, such as IRS-1, IRS-2, and Src homology C terminus (SHC), by phosphorylation. This causes activation of additional kinases, phosphatases, and other signaling molecules leading to a complex pathway divided into two major signaling pathways [9].

The first signaling pathway is the metabolic pathway. Activation of phosphatidylinositol-3-kinase resulting from binding to a phosphorylated IRS causes movement of vesicles containing glucose transporter 4 (GLUT-4) to the cell membrane. These events allow increased cellular glucose uptake down a concentration gradient and prevent a substantial postprandial rise in circulating glucose concentration. The second pathway is the mitogenic pathway that regulates growth effects of insulin. Binding of SHC proteins to either the insulin receptor or IRS protein leads to activation of SHC. This ultimately results in increased gene expression [9].

Insulin is a key regulator of carbohydrate metabolism. In the postprandial period, glucose is absorbed into portal circulation from the small intestine. The elevation in glucose in

portal blood triggers pancreatic release of insulin. Subsequently, insulin increases entry of glucose into muscle, liver, and adipose tissue via GLUT-4 transporters. In muscle, protein synthesis is also increased in the presence of insulin by increasing amino acid uptake and ribosomal protein synthesis. In the liver, insulin also activates glycogen synthesis and storage and inhibits breakdown of glycogen post-prandially by decreasing activity of glucose-6-phosphatase. Appropriate insulin action maintains the plasma concentration of glucose within a narrow range in order to prevent disease. Further, following a meal, insulin release also provides a signal for satiety [9,13].

Insulin also regulates lipid metabolism. Triglycerides are energy dense storage molecules and their production and storage in adipose tissue is increased in response to insulin. Additionally, insulin inhibits hormone-sensitive lipase that hydrolyzes triglycerides to fatty acids, therefore, limiting breakdown of fat. When insulin production is decreased, for example during a fasting state, hormone-sensitive lipase is strongly activated promoting hydrolysis of triglycerides and release of glycerol and non-esterified fatty acids (NEFAs) into circulation for gluconeogenesis and β-oxidation to provide energy [13].

Insulin Resistance, Diabetes Mellitus, and Metabolic Syndrome

Insulin resistance is a metabolic disturbance defined as a decrease in the uptake of glucose by the liver, skeletal muscle, and adipose tissue as a result of reduced tissue sensitivity to insulin. Insulin resistance involves a failure of insulin signaling [62]. This failure can be due decreased numbers of insulin receptors on cell surfaces, decreased activation (downregulation) of insulin receptors following insulin binding, or altered triggering of downstream intracellular pathways that lead to translocation of GLUT-4 transporters to the plasma membrane [114]. Insulin resistance has been described as either compensated or uncompensated. Compensated

insulin resistance is characterized by greater postprandial insulin secretion by the pancreas to appropriately control blood glucose concentration. Maintenance of normoglycemia is accompanied by intermittent or persistent hyperinsulinemia. If pancreatic beta cells eventually become "exhausted" from overproduction of insulin and can no longer produce adequate amounts of insulin, intermittent or persistent hyperglycemia may develop. This condition is termed uncompensated insulin resistance and can be accompanied by either high or low circulating insulin concentrations [74].

Insulin resistance is a component of many metabolic disorders and is a risk factor for a number of medical problems in humans and domestic animals. In humans, these include obesity, dyslipidemias, type 2 diabetes mellitus, microalbuminuria, and cardiovascular problems including hypertension (collectively termed Metabolic Syndrome [MS]) [1]. In the equine population, insulin resistance is also recognized with obesity or regional fat deposition, hyperlipidemia, hypertension (less well documented than in humans), and diabetes mellitus (much rarer in equids than humans). Due to similarities to the human condition, the term Equine Metabolic Syndrome (EMS) was coined to describe this cluster of medical problems in equids [56]. However, the primary clinical problem affecting equids with EMS is development of insidious onset laminitis, often accompanying ingestion of excessive amounts of soluble CHOs, notably lush pasture [39].

In humans, diabetes mellitus (DM) is the most common metabolic disease characterized by persistent hyperglycemia resulting from reduced insulin secretion and/or insulin resistance. Type 1 DM most commonly develops in children as a consequence of immune-mediated pancreatic beta cell destruction resulting in decreased insulin production [74]. In addition to a lack of insulin causing decreased glucose uptake by tissues, the liver produces additional glucose

and ketones. Both hyperglycemia and increased ketones produce an osmotic diuresis. Also, metabolic acidosis can develop as a consequence of increased production of ketones. Treatment for type 1 DM requires administration of exogenous insulin to control hyperglycemia. If a patient is not treated with insulin, they may die of diabetic ketoacidosis [9,13]. In the horse, this type of DM is rare [56].

Pancreatic beta cell "exhaustion" or failure following long-standing insulin resistance leads to the development of type 2 DM [62]. Individuals with type 2 DM can produce some insulin but pancreatic output to an increase in plasma glucose is inadequate to maintain normglycemia. Thus, both insulin secretion and glucose homeostasis are abnormal in affected patients. The primary treatment for patients with this type of DM is diet and exercise in order to better control blood glucose levels. It is recommended that patients eat well-balanced meals on a regular schedule to avoid extremes in fluctuation of blood glucose concentration. Increasing the fiber content in the diet can also attenuate fluctuations in blood glucose by slowing the rate of CHO absorption from the intestine. If diet and exercise alone do not adequately control blood glucose concentration, medications to lower blood glucose concentration and/or insulin therapy may be prescribed [13]. In the human population, type 2 DM is generally diagnosed in adulthood and is seen more frequently in obese individuals [9]. Although insulin resistance is common in obese equids, progression to type 2 DM is a rare occurrence. In fact, type 2 DM is seen most frequently in older equids with insulin resistance as a component of pituitary pars intermedia dysfunction [23].

Metabolic syndrome is a clustering of medical disorders that increase the risk of type 2 DM and cardiovascular disease. Other names for MS include insulin resistance syndrome and syndrome X [56]. There a multiple criteria required to establish a diagnosing of metabolic

syndrome. The World Health Organization's criteria for diagnosing MS include presence of impaired glucose tolerance/insulin resistance or overt type 2 DM, along with two of the following abnormalities: hypertension, dyslipidemia, central obesity, or microalbuminuria [1]. Development of this complex disease syndrome is not completely understood but risk factors include diet, obesity, genetic predisposition, aging, stress, and a sedentary lifestyle [35]. As previously mentioned, EMS is the term commonly used to describe a similar cluster of metabolic disorders in equids [56]. Although pathophysiology may not be identical in both species, overfeeding of CHO, genetic predisposition, and a sedentary lifestyle also appear to be important risk factors for development of EMS in equids. However, development of laminitis is a clear clinical difference between patients with MS and EMS and the pathophysiologic processes that culminate in this painful and debilitating lameness remain incompletely understood.

Measurement of Insulin Sensitivity

Because insulin resistance is a key metabolic disorder in both MS and EMS, measuring tissue sensitivity to insulin has been a strong focus of both research into the syndromes and managing affected patients. Tests to measure insulin sensitivity can be divided into non-specific (indirect) and specific (direct) assessment of insulin resistance. Measurement of basal circulating glucose and insulin concentration is the simplest non-specific test. Next, various mathematical manipulations of these two measurements, termed proxy measurements, have been performed in an attempt to improve diagnostic sensitivity and specificity of these measurements. Measurement of changes in glucose and insulin concentrations in response to enteral or intravenous glucose (or glucose and insulin) administration is additional tests that provide dynamic assessment of pancreatic insulin production and tissue glucose uptake but they do not directly assess tissue insulin sensitivity. There are three specific or direct, quantitative tests that

have been used to measure tissue insulin sensitivity: the euglycemic-hyperinsulinemic clamp, the frequently sampled intravenous glucose tolerance test (FSIVGT) with minimal model analysis, and the insulin suppression test [62].

Non-specific measurement of insulin sensitivity

Measurement of basal (fasting) glucose concentration is a common screening test for DM in human medicine [62]. Fasting hyperglycemia indicates that there is either a decrease in tissue glucose uptake due to decreased insulin sensitivity or that pancreatic beta cells are secreting inadequate insulin to maintain normoglycemia [31]. Thus, this measurement does not determine a definitive cause for hyperglycemia. Further, glucose concentrations within the blood can vary substantially over a short period of time due to environmental factors such as stress, feeding, and diurnal variation [113]. Despite limited use to assess tissue insulin sensitivity, measuring glucose concentration remains a simple and cost-effective test in human medicine where DM is more commonly recognized than in equids.

Measurement of basal (fasting) insulin concentration is another simple test and values are inversely correlated with insulin sensitivity [106]. A fasting insulin concentration of $\geq$20 mU/L in equids is defined by the American College of Veterinary Internal Medicine Consensus Statement as supportive of insulin resistance [35]. Hyperinsulinemia develops when more insulin is secreted due to decreased tissue sensitivity to insulin (compensated insulin resistance). Hyperinsulinemia may be persistent or may only be detected for a few hours post-prandially; therefore, false-negative (normal) results may be found in fasting samples in equids with insulin resistance. In theory, persistent hyperinsulinemia would seem to support more severe insulin resistance than intermittent postprandial hyperinsulinemia but this has not been proven in equids

and, similar to glucose, insulin concentration can change with stress and other external factors [62,113].

Various proxies, or mathematical manipulations of glucose and insulin concentrations measured in the same fasting blood sample, have been developed in an attempt to better assess pancreatic function and insulin sensitivity. In human medicine, proxies have been validated in large patient populations and can more useful in identifying insulin resistance that glucose or insulin concentrations alone [112]. One example is the glucose-to-insulin ratio that correlates positively with insulin sensitivity. Additionally, the insulin-to-glucose ratio is positively correlated with insulin secretion [62]. The insulin-to-glucose ratio has been found to decrease within 15 minutes after intravenous injection of glucose in ponies and horses with pituitary pars intermedia dysfunction (PPID) when compared to normal horses [37]. Also, a modified insulin-to-glucose ratio (MIRG) and the reciprocal of the square root of insulin (RISKI) values have been reported to be useful to identify ponies with pre-laminitic metabolic syndrome [113]. Although simple to calculate, validation of these proxies in equine medicine remains limited as they have only been evaluated in small groups of equids.

The glucose tolerance test is another non-specific measure of insulin resistance. This test involves serial measurement of glucose concentration following enteral or intravenous administration of glucose. The time required for plasma glucose concentration to return to baseline is indicative of the subject's ability to assimilate (absorb, utilize, and store) glucose [31]. Glucose intolerance, indicated by either a greater increase or longer duration of hyperglycemia as compared to normal subjects, can be a consequence of inadequate pancreatic production or secretion of insulin or impaired ability of tissue glucose uptake. Thus, these tests only provide indirect support of tissue insulin sensitivity [62].

The oral glucose tolerance test was introduced in equine medicine in 1973 to diagnose small intestinal disease and to assess pancreatic endocrine function [95]. Following an overnight fast, horses are administered glucose (typically 1 g/kg) via a nasogastric tube or oral dosing syringe. A baseline blood sample is collected prior to glucose administration followed by blood collection every 30-60 minutes for 6 hours following glucose administration. Blood samples are then analyzed for glucose and insulin concentration. Glucose concentration typically peaks from 90-120 minutes and should return to the baseline value after 4 hours [31]. When compared to an intravenous glucose tolerance test, variation in gastric emptying and intestinal absorption makes interpretation of oral glucose tolerance test results difficult for assessment of tissue insulin sensitivity, although an excessive rise in insulin concentration provides indirect support [91]. Also, stress accompanying nasogastric tube placement can cause transient increases in blood glucose and insulin concentrations [31]. Although it can provide support for insulin resistance , the oral glucose tolerance test has more commonly been used as a diagnostic aid to document small intestinal malabsorption syndromes in horses [68].

Recently, however, there has been renewed interest in an "oral sugar test" as a practical and useful screening tool to document insulin resistance in equids. In this test Karo Syrup Lite™ is administered orally (15 mL/100 kg = 5 g sucrose) and blood samples are collected prior to dosing and 75 min after administration. An exaggerated insulin response ($\geq$60 mU/L at 75 minutes) is supportive of tissue insulin resistance [99].

The intravenous glucose tolerance test eliminates gastrointestinal factors that must be considered with the oral glucose tolerance test [62]. The intravenous glucose tolerance test requires an intravenous catheter to be placed in the jugular vein a few hours prior to the test. The animal should be fasted for at least 12 hours prior to receiving a rapid bolus of glucose (0.1-0.3

g/kg) intravenously. Blood glucose and insulin concentrations are determined from blood samples collected from time zero to 4 hours post-glucose infusion [31]. Peak glucose concentration is typically measured in the initial post-administration sample (15 minutes) with a decline to the baseline value within 1 hour. Insulin concentration peaks at 30 minutes and return to baseline following the pattern of the glucose response curve. If the blood glucose peak is either higher at 15 min, when compared to normal horses, or does not return to baseline values within 1 hour, test results support decreased tissue sensitivity to insulin [37,91]. However, to better document insulin resistance, insulin concentrations should also be measured to determine whether the pancreas in not producing enough insulin (low insulin concentrations) or the peripheral tissues are not responding to insulin (high insulin concentrations) [31].

The insulin tolerance test is another non-specific assessment that has been used to document insulin resistance. This test measures the blood glucose response to an intravenous dose of insulin. In a normal animal, blood glucose concentration falls to approximately 50% of the baseline value within 30 minutes of insulin injection. Blood glucose concentration should return to normal within 1.5-2 hours. If the animal is insulin resistant, the decrease in blood glucose concentration following insulin administration will be blunted [31].

The combined glucose-insulin tolerance test (CGIT) was subsequently developed to assess the effects of simultaneous administration of glucose (0.15 g/kg) and insulin (0.1 U/kg), after an overnight fast. The CGIT essentially couples the dynamic responses observed in both the glucose tolerance and insulin suppression tests. Blood glucose is measured serially over the next 3 hours and results typically show an initial period of hyperglycemia followed by a period of hypoglycemia. Variation in the magnitude of these responses (excessive hyperglycemia or blunting of hypoglycemia) is supportive of reduced tissue sensitivity to insulin [27].

Specific measurement of insulin sensitivity

Three specific, quantitative methods have been used to determine tissue sensitivity to insulin. The first method is the insulin suppression test that measures the effect that insulin has on plasma glucose concentration when insulin and glucose are infused at a fixed rate. First, an inhibitory hormone, typically somatostatin is administered intravenously at a constant rate throughout the test to inhibit release of endogenous insulin from the pancreas and to suppress hepatic glucose release. Next, insulin and glucose are also infused intravenously at a constant rate. After an equilibration period, the infusion rate is manipulated during the last 60 minutes of the 150 minute test to produce steady-state glucose and insulin concentration. Mean glucose infusion rate during this time period is a measure of insulin-mediated glucose disposal rate that directly correlates with tissue insulin sensitivity [62]. The purpose of this test is to limit the effects of endogenous insulin and hepatic glucose production in order to specifically measure the effect of exogenous insulin on tissue glucose uptake [100]. Difficulties with the insulin suppression test, including adverse reactions to somatostatin, challenges in maintaining steady-state glucose and insulin concentrations, and development of hyperglycemia and glucosuria, have limited both clinical and research use of this test [62].

The second quantitative method is the euglycemic-hyperinsulinemic clamp. During this test hyperinsulinemia is induced by an initial bolus followed by a constant rate intravenous infusion of insulin and euglycemia is maintained by adjusting the intravenous infusion rate of glucose through a catheter in the contralateral jugular vein [30]. Insulin infusion suppresses endogenous glucose production and the goal of the test is to maintain a physiologic glucose concentration, typically 5 mmol/L, in the face of hyperinsulinemia. In order to maintain this glucose concentration, frequent blood samples are collected and immediately analyzed in order

to adjust the glucose infusion rate appropriately. After an initial equilibration period of 60-120 minutes, mean glucose infusion rate during the last 60 minutes of the test is a measure of glucose disposal rate. A low glucose disposal rate indicates reduced tissue sensitivity to insulin or insulin resistance [62].

Although considered by many researchers to be the "gold standard" test for measuring tissue insulin sensitivity, the euglycemic-hyperinsulinemic clamp technique has several limitations. First, it is an intensive technique that requires skilled assistants. Next, multiple intravenous catheters must be placed, for sample collection and for insulin and glucose infusions. Further, it has been argued that the test is non-physiologic in nature because the insulin concentration achieved is often above the physiologic range [29]. Finally, the relationship between glucose disposal rate and concentrations of glucose and insulin is complex and could lead to substantial variation under different experimental conditions [44,62]

Minimal model analysis of insulin and glucose dynamics during a FSIVGT is the third quantitative test used to directly measure insulin resistance. The goal of this test is to assess insulin and glucose dynamics under physiologic insulin concentrations. Minimal model analysis also allows estimation of additional parameters including the acute pancreatic insulin release in response to an intravenous bolus of glucose, glucose effectiveness, and the disposition index [49]. The procedure involves placing bilateral jugular intravenous catheters, one for glucose and insulin administration and one for sample collection, usually the night before the test to limit any stress of catheter placement on test results. Subjects are also fasted overnight. On the day of the test, a bolus of glucose (0.1-0.3 g/kg) is administered followed by a bolus of insulin (10-20 mU/kg) 20 minutes later. Approximately 25 blood samples are collected over the subsequent 4-6 hours and glucose and insulin concentrations are subjected to minimal model analysis [62].

Minimal model analysis uses two differential equations to describe glucose-time curves, dividing glucose disposal into two phases: glucose and insulin-mediated disposal. The first equation calculates glucose-mediated glucose disposal using a single-rate constant. The result provides an estimate of glucose utilization (uptake) following the intravenous bolus of glucose. The second equation calculates insulin-mediated glucose disposal by using a rate constant that incorporates insulin sensitivity. The result provides an estimate of the ability of insulin to remove glucose from the blood by decreasing endogenous glucose production and increasing the use of glucose by tissue, in response to the bolus of exogenous insulin [62]. Limitations of minimal model analysis include an argument that a single-rate constant in the first equation may be inadequate to model the complex interaction of glucose and insulin. Next, another concern has been that hyperglycemia in response to the glucose bolus could exceed the renal threshold and lead to loss of glucose in the urine during the first 20 minutes of the test. The minimal model does not account for this and, as a consequence, the test has been modified by lower the dose of glucose administered to 0.1 g/kg [109].

Glucose effectiveness (Sg), insulin sensitivity (Si), acute insulin response to glucose (AIRg), and the disposition index (Di) are parameters calculated using minimal model equations. Glucose effectiveness is an estimate of the ability of glucose to drive its own disposal (tissue uptake) without the influence of insulin. Insulin sensitivity is a measure of the ability of insulin to promote glucose disposal. Acute insulin response to glucose measures the endogenous insulin output by the pancreas in response to glucose administration. Lastly, disposition index is the product of acute insulin response to glucose and insulin sensitivity and provides an assessment of beta cell responsiveness to glucose [49].

Along with comparing peak values and general shape of the curves, the statistical analysis of the various glucose and insulin tolerance tests described above includes calculating area under the curves for plasma glucose and serum insulin. These values are calculated with commercially available computer software using trapezoidal integration [115]. The value of the area under the curve can be used to compare different experimental conditions as it is a representation of overall height and width of the glucose or insulin response. In human medicine, it has been shown that there is an increase in the area under the curve for glucose when a high-starch diet is consumed [17]. In equine research, area under the curve was used to determine the appropriate dosage of dextrose to be administered during the frequently sampled intravenous glucose tolerance test to minimize urine glucose spilling [109].

Factors Affecting Insulin Sensitivity

There are certain factors which affect insulin sensitivity. Age has been shown to affect insulin sensitivity throughout certain stages in life. In a newborn foal, for the first 24 to 48 hours, the beta cells within the pancreas are still maturing which leads to transient insulin resistance [52]. This maturation of beta cells within the pancreas continues for approximately three months post-parturition at which time the pancreatic cells are functioning at the level of a mature horse [103]. In human children at the onset of puberty, there is a stage of insulin resistance which corresponds with a time of rapid development and growth [88].

Human literature has reported that glucose tolerance deteriorates with age. Using an oral glucose tolerance test in healthy men, peripheral glucose uptake was 3 times greater in young and middle-aged men as compared with elderly men. Glucose and insulin responses were similar in all three age groups which suggest that the impairment in the elderly is not at the level of the insulin but a result of impairment in downstream mechanisms of glucose metabolism [53].

When using the euglycemic-hyperinsulinemic clamp at different levels of glycemia in elderly versus nonelderly subjects, the glucose disposal rate was decreased by 30-35 % in the elderly at each level of glycemia with similar levels of hyperinsulinemia. This study reported similar affinity of glucose utilization indicating that there was no impairment in the affinity for the glucose receptor but rather a reduction in the number of functional receptors [30]. In conclusion, aging can affect insulin resistance by both reduced glucose uptake with fewer receptors and impaired intracellular glucose metabolism [43].

Limited research has been conducted investigating the effect of age on insulin sensitivity in the horse. In 2002, following a 0.25 g/kg of body weight oral glucose tolerance challenge, old Standardbred mares (27.0 ± 0.4 yrs) had a significantly greater insulin response as compared to middle-aged (15.2 ± 0.4 yrs) and young (6.8 ± 0.4 yrs) Standardbred mares [69]. Furthermore in 2010, sixteen Arabian horses, eight 2-year olds and eight mature horses (14 ± 0.5 year), underwent weekly glycemic response tests using various feedstuffs. Despite minimal differences in glucose responses between 2-year olds and mature horses, mature horses had a greater insulin response suggesting that mature horses have reduced insulin sensitivity [83]. When comparing old (22 years ± 0.7 yrs) and young (7 years ± 0.6 yrs) Standardbred mares using the frequently sampled intravenous glucose tolerance test, old horses had a greater AIRg suggesting a greater pancreatic output of insulin in response to the glucose challenge [64]. In both humans and horses, it has been shown that insulin sensitivity decreases with age, leading to an increased prevalence of insulin resistance [80].

The mechanisms of digestion post-prandially are also influenced by age. In humans, there seems to be a higher prevalence of gastrointestinal disorders of function and motility with aging [96]. It has been reported that peristalsis and gastric contractile force are reduced in aged

individuals following a meal. Lipids also have been shown to delay gastric emptying time in elderly subjects [82]. This delay in gastric emptying may also be a result of a reduction in the number of myenteric neurons available to signal neurotransmitter responses [96]. Aging increases the proliferation of gastric mucosa, however, this mucosa is reported to be more susceptible to injury [78]. The number of gastric and colonic mucosal cells undergoing apoptosis increased with a restriction in caloric intake [126]. Also, the responsiveness of functional receptors for peptides such as gastrin is reduced with age [96]. This suggests that aging influences nutritional and hormonal impacts on gastric mucosa. Disease can have major effects on intestinal absorption including atrophic gastritis. *Helicobacter pylori* infections are associated with a decrease in gastric acid secretion [78]. Consequences of atrophic gastritis include bacterial overgrowth in the proximal intestinal tract and malabsorption [96]. While the effect of age on digestion has been researched extensively in the human literature, very little has been reported in the horse.

As the horse ages, dentition becomes an important management factor. The process of digestion begins in the mouth with thorough mastication of a primarily fibrous diet. A sufficient surface area is needed for proper mastication of feed. For grinding forage, the horse has cheek teeth that form solid arcades of enamel ridges. The horse has hypsodont, or low-crowned, teeth which erupt throughout life until approximately twenty years of age. Around this age, the reserve crown is shortened to the extent that that tooth is shed [42]. This reduces the circumference of the tooth which allows for diastemas, spaces between teeth, to form. Additionally, the surface area for grinding is reduced [65]. The type of diet affects the dentition of the horse as well. Horses fed a primarily forage diet have a greater lateral excursion of the arcades and a larger amount of occlusal surface to contact the opposite tooth than when fed a

high-concentrate diet. Reduced occlusal wear from a high-concentrate diet also restricts the lateral chewing action of the horse [42]. Aging and diet effect the dental health of the horse which influences the animal's ability to digest a primarily forage diet.

Another trait influencing insulin sensitivity is breed. It has been documented that ponies are less sensitive to insulin than Standarbred horses [55]. In support, a study reported that donkeys are less insulin sensitive than ponies or horses [58]. In humans, certain ethnicities are more susceptible to developing insulin resistance. For example, the prevalence of metabolic syndrome in Mexican Americans is significantly higher (32%) than non-Hispanic whites (24%) and non-Hispanic blacks (22%) [93]. More specifically, black South African women are more insulin resistant with a lower insulin sensitivity and higher acute insulin response to glucose than white South African women following minimal model analysis [41]. Lastly, it was shown that African-American women have lower insulin sensitivity than European-American women [44]. Specific horse breeds have been identified as having a greater risk of developing insulin resistance. These breeds include Morgan Horses, European Warmbloods, American Saddlebreds, Spanish mustangs, and all pony breeds. One recent study comparing 8 Standardbred, 8 mixed-breed ponies, and 7 Andalusian-cross horses showed that ponies and Andalusian horses have a lower insulin sensitivity and AIRg as compared to Standardbreds [5]. In summary, there appears to be a genetic predisposition impacting insulin sensitivity.

Pregnancy can also have an effect on insulin sensitivity. In humans, insulin resistance during pregnancy, known as gestational diabetes, is an important risk factor leading to abortion [70]. Pregnant mares have a significantly higher basal insulin and greater insulin response to an intravenous glucose challenge as compared to non-pregnant controls at less than 270 days of gestation [34]. Additionally, there was a significantly greater insulin secretion at 28 weeks of

gestation in pregnant mares versus non-pregnant mares following minimal model analysis [40]. Fetal growth rate has a negative consequence on insulin sensitivity as well. Pony fetuses transferred to Thoroughbred mares during gestation have higher basal insulin levels and greater beta cell response to glucose when allowed to grow larger in utero than normal [33]. This suggests that factors related to pregnancy do have an impact on insulin sensitivity of both mother and offspring.

Obesity is very important risk factor influencing insulin sensitivity. Obesity generally results from an imbalance between the energy consumed and lost. Through evolution, horses have "thrifty genes" that allow for improved feed efficiency during harsh environmental conditions. These genes are detrimental to the majority of horses that live a sedentary life with minimal controlled exercise. The daily energy requirement of such horses is typically less than what the horses are actually fed whether the horse is stalled or on pasture. This results in excessive caloric intake and deposition of adipose tissue [39]. In the horse, obesity can be determined by body condition scoring using the Henneke scale (1-9). Horses with a score of 8 or 9 are generally considered obese [47]. It has been shown in a variety of species including mice, dogs, humans, and rats, overfeeding and accumulation of adipose tissue results in a decreased insulin sensitivity [11].

Adipose tissue regulates metabolism by releasing inflammatory cytokines, hormones such as leptin and adiponectin, non-esterified fatty acids (NEFAs), and glycerol. Obesity induces a chronic inflammatory state by increasing the production and circulation of these products which have been associated with insulin resistance [127]. The exact mechanism is unclear, but it is thought that interference with insulin signaling pathways leads to decreased insulin sensitivity. In previous research comparing lean and obese horses, the results showed

that the obese horse has lower insulin sensitivity as well as higher plasma concentrations of insulin, glucose, leptin, and NEFAs. In this study, a 20% increase in body weight resulted in compensated insulin resistance [11]. Other research in the horse has shown that increased body condition and percent fat are associated with increased expression of TNF-α and IL-1 which may inhibit insulin action [117].

In human literature, it has been shown that within hours of an acute increase in NEFA levels in plasma, the development of insulin resistance occurred. Obesity increases the production of adipokines which are cytokines released from adipose tissue that cause insulin resistance [66]. Also, adipocytes contain an enzyme, 11-β hydroxysteroid dehydrogenase (11-β-HSD1), which converts cortisone to cortisol. Cortisol opposes the effects of insulin on carbohydrate metabolism causing a decrease in glucose uptake by peripheral tissues and increased production of glucose by the liver [71]. By examining transgenic mice that overexpress 11-β-HSD1, there was an increased development of visceral obesity, insulin resistance, type 2 diabetes, and hyperlipidemia [56]. Lastly, as a result of adipose tissue reaching maximum storage capacity with excessive glucose being converted to fat, lipotoxicity in other tissues can occur causing alteration in normal cellular function including insulin resistance [35].

Obesity is a causative factor leading to a variety of health disturbances in humans and domestic animals. In the horse, insulin resistance in conjunction with obesity has been associated with an increased prevalence of laminitis [39]. However, it has not been determined whether this is a direct correlation or whether obesity increases the prevalence of factors such as insulin resistance and inflammation leading to laminitis. Insulin is a vasoregulatory hormone using nitric oxide to invoke vasodilation. When there is a decrease in the tissue sensitivity to insulin in the lamina of the hoof, there is a reduction in vasodilatory effects of insulin resulting in

decreased blood flow to the hoof. Deprivation of nutrients such as glucose to the lamina can lead to separation [35]. Also, keratinocytes of the lamina have glucose transporters which are reliant on insulin for regulation of hoof growth [111]. In the equine literature, it has been shown that prolonged hyperinsulinemia and euglycemia triggered laminitis within 72 hours in healthy, moderate body condition ponies with no history of laminitis [4]. To further support this finding, experimental induction of hyperinsulinemia triggered laminitis in young (3-4yr), non-obese Standardbred horses within 48 hours [19]. Therefore, a reduction in the insulin sensitivity of the hoof and hyperinsulinemia are risk factors for the development of laminitis.

Physical activity has been shown to improve insulin sensitivity in many species of animals. In both humans and rats, short periods of acute exercise improved insulin action [48,59]. In the horse, exercise training decreased the acute insulin response to glucose (AIRg) in young Standardbreds [64]. Additionally, in both obese and lean mares, insulin sensitivity improved, 60% and 48% respectively, following acute exercise training with no change in body weight. Nine days post exercise training, there was no difference in insulin sensitivity from baseline suggesting physical activity caused an acute improvement only [89].

Diet can influence the prevalence of insulin resistance. Carbohydrate restriction has been shown to improve all markers of metabolic syndrome in humans [118]. A low-carbohydrate diet for fourteen days was reported to improve insulin sensitivity by 75% [8]. Following a 56 week diet adaptation to a ketogenic (high fat, low carbohydrate) diet, there was a significant reduction in blood glucose in 31 obese, diabetic patients [15]. In order to reduce the glucose content of the diet, the total carbohydrate content as well as the type of carbohydrate must be considered. Starches are 100% glucose whereas sucrose is approximately 50 % glucose. Substituting sucrose or lactose for starch in a single meal was reported to reduce the glucose area response by

approximately 40-50% [60]. Dietary protein does not increase blood glucose concentration but does increase insulin secretion. Therefore, consumption of protein with ingestion of glucose will reduce the blood glucose response to the glucose ingestion by increasing insulin secretion [36]. When adapted to a high–monounsaturated fat/low-carbohydrate diet, Type 2 diabetic patients showed significant decrease in postprandial plasma glucose and insulin. Additionally, using euglycemic-hyperinsulinemic clamp, a significantly higher insulin-mediated glucose disposal was documented suggesting an increased uptake of glucose by peripheral tissue or improvement in insulin sensitivity [86]. In summary, dietary adjustments including limiting carbohydrate intake and increasing protein intake can improve tissue sensitivity to insulin in humans.

As a non-ruminant herbivore, the horse is well adapted to a diet high in carbohydrates. There are two main sources of carbohydrates in the equine diet: structural carbohydrates (SC) and nonstructural carbohydrates (NSC). Simple sugars, starches, and fructans are examples of NSC while cellulose and hemicelluloses are SC [35]. In general, the fibrous part of the diet constitutes the SC content while the high-energy yielding concentrate portion comprises the NSC content. It should be noted that the forage species in some pastures can contain a high NSC content. Additionally, growing conditions, maturity when harvested, and drying conditions all effect the amount of NSC in hay. Forage analysis is a method used to determine the exact amount of NSC in hay [46].

The type of carbohydrate is also described by a measurement of glycemic index. The glycemic index of a feed is the plasma glucose response to consumption of a measured amount of feed when compared to a standard challenge. In humans, this standard challenge is a meal consisting of white bread. In the equid, the standards vary from an equivalent weight of oats to an oral dose of glucose or a standardized amount of NSCs in the feed. Hay is generally

classified as having a low glycemic index whereas grains have a higher glycemic index [92]. In general, foods that produce a higher peak and greater overall glucose response are considered to have a higher glycemic index [55] and can range from 40-60% NSC on a dry matter basis [106]. When comparing types of grain supplementation, a starch and sugar concentrate (sweet feed) has a higher glycemic index (> 30 % NSC) than a high fat and fiber concentrate (<20 % NSC) [92].

The effect of diet on glucose metabolism was first recognized by Argenzio and Hintz in 1972. Greater glucose availability and improved glucose utilization was seen when ponies were adapted to an oat diet compared with a high fiber diet of alfalfa and beet pulp [3]. Jacobs and Bolton further explored the effect of diet on glucose metabolism in 1982 by using the OGTT. Horses adapted to a diet of oats and alfalfa hay had a significantly lower glycemic response when compared with horses on a pasture diet [54]. The OGTT was also used to document a significantly lower glycemic response in ponies adapted to a high fiber pellet diet versus a hay only diet [80]. A lower area under the curve and reduced insulin sensitivity has been documented in horses adapted to a starch and sugar diet as compared to a fat and fiber diet [90]. In healthy Thoroughbred geldings adapted to a high starch and sugar feed versus a fat and fiber rich feed, there was a decrease in insulin sensitivity when adapted to the starch and sugar diet. Also reported in this study was a lower acute insulin response to glucose and disposition index. This was not an expected finding as acute insulin response to glucose typically compensates for lower insulin sensitivity by increasing in value. This finding suggests that there was a reduced beta-cell response when adapted to the starch and sugar diet [50]. When comparing the insulin and glucose concentrations post-prandially, horses fed a starch and sugar grain had significantly higher plasma concentrations then horses fed a fat and fiber diet [50, 112]. In support, Thoroughbred weanlings adapted to a high glycemic meal had a 37 % decrease in insulin

sensitivity when compared to a fat and fiber feed. Furthermore, the decreased insulin sensitivity was compensated with an increase in the acute insulin response to glucose [112]. Lean horses fed three diets differing in NSC content showed no difference in glucose and insulin response within treatment suggesting no alteration in peripheral glucose disposal following a 90 day diet adaptation. Interestingly, AUCg was significantly lower, peak insulin concentration was significantly greater, and AUCi did not change on day 90 when compared with day 0 [106]. An explanation for these results is unknown as a decreased AUCg and increased peak insulin do not allow for distinguishing between increased tissue sensitivity to insulin versus reduced insulin sensitivity. Furthermore, a reduction in insulin-induced adipose tissue lipolysis was reported on high NSC diet indication diet associated insulin resistance. In a different animal model, rats fed a high glycemic index starch developed more body fat when compared to rats fed a low glycemic index starch. This suggests that the high glycemic index meal which induces hyperinsulinemia increases the amount of nutrients deposited as fat [87].

Meals with a high glycemic index can pose a variety of problems in the horse. Chronic adaption to meals with a high glycemic index causes fluctuations in glycemia and insulinemia. In previous research, it was shown that the main energy source of the equid changes in the spring from stored fat to soluble carbohydrates. This switch is associated with an increased prevalence of insulin resistance and increased risk of laminitis [113]. It has been shown in horses that the greater NSC content of a meal, the higher the insulin and glucose response which may potentiate the development of insulin resistance [116]. Horses given a 6-hour insulin infusion as a model for a horse adapted to a high NSC diet had a decreased abundance of GLUT-4, GLUT-1, and insulin receptor in adipose tissue while GLUT-1 was increased and insulin receptor was

decreased in skeletal muscle. Glucose and lipid transport was affected by hyperinulinemia in this study promoting insulin resistance [105].

Large meals consisting of NSC increase the rate of gastric emptying and gut transit time while decreasing digestion of the starch and sugars in the small intestines before reaching the hindgut. Disturbances in the micro flora and fermentation of the hindgut with a diet overload of carbohydrates results in an increase in intestinal permeability [77]. This can induce acidosis of the hindgut of the equid as a consequence of undigested starch being rapidly fermented by gram positive bacteria. This allows for an influx of endotoxins, resulting in inflammatory responses including onset of laminitis, increased risk of colic and higher risk of effecting mineral content of bone [52, 92].

Replacing high NSC concentrates such as sweet feed with a fat and fiber feed has been a popular interest in research due to the possible benefits in the horse industry such as reducing rhabdomyolysis, enhancing both anaerobic and aerobic exercise and reducing excitability [125]. When looking at glucose and insulin dynamics, feeding a high fat and fiber diet results in an increase in insulin sensitivity and reduced hyperinsulinemia and more closely mimics the natural state of a grazing horse versus a high starch diet [50, 92]. Compared to sweet feed and a pelleted concentrate, a feed high in fat and fiber had the same energy density but with an insulin response of a typical alfalfa hay [125]. Following a six week diet adaptation period, young, healthy Standardbred horses exhibited no change in insulin sensitivity or glucose tolerance on the fat and fiber diet versus when adapted to a high NSC concentrate diet where insulin sensitivity and glucose tolerance were decreased [90]. In humans, both high fat and high carbohydrate diets are known to cause insulin resistance [55].

Equine metabolic syndrome is being researched heavily today as the direct cause and effect relationships of this syndrome are not completely understood but are very extensive and intertwined. In particular, limited research has been devoted to the effect that age has on the development of equine metabolic disease. Additionally, there is confusion as to what feed is appropriate for the aged or senior horse. There are a variety of different concentrates marketed to both the senior and obese horse including low-starch and senior feeds. Senior feeds are high in starch which has led to veterinarians to recommend low-starch feeds to the aged horse due to the perceived increase in insulin resistance with age. However, low-starch feeds are not a complete feed which is problematic for the aged horse with poor dentition. This has led to some confusion as to what the appropriate diet is for the aged horse. Therefore, the purpose of the following study was to investigate the effect of diet on age-related changes in glucose and insulin dynamics in the horse.

CHAPTER 2

EFFECTS OF AGE AND DIET ON GLUCOSE AND INSULIN DYNAMICS IN THE HORSE

Introduction

Factors affecting insulin sensitivity in equids include breed, body condition, pregnancy and lactation, physical activity, age, and diet [5, 6, 31, 35, 72]. With respect to the latter two, ageing is associated with development of glucose intolerance and insulin resistance (IR) in people, manifested by exaggerated glucose and insulin responses to carbohydrate (CHO) challenge [20, 30, 53]. Greater insulin responses to enteral glucose challenge [69], as well as to cereal grain meals rich in hydrolysable CHO [83], have also been documented in mature horses, as compared to young horses. These findings support an age-related decrease in insulin sensitivity in this species as well. Importantly, IR, especially when coupled with genetic predisposition and obesity, is considered a risk factor for development of medical problems including cardiovascular diseases, dyslipidemias, and type 2 diabetes in people [85] and laminitis in equids [35, 45, 72, 75].

Diets high in nonstructural CHOs (NSC) have also been implicated in development of IR and type 2 diabetes in people, even after controlling for other risk factors [73]. Equids are usually fed forage diets that are relatively low in NSC. Similar to people, when horses ingest larger amounts of NSCs, either in lush pasture grass or when forage is supplemented with cereal grains, greater postprandial glucose and insulin responses, along with decreased insulin sensitivity, have been documented [11, 49, 83, 90]. Ingestion of diets high in NSC may further affect glucose and insulin dynamics by altering the enteroinsular axis, consisting of enteric neuronal signals and gut-derived hormones (incretins) that influence pancreatic insulin release [6,18]. As a

consequence, when equids require greater caloric intake to meet demands of exercise or lactation, use of oil-fortified feeds has been recommended to decrease the risk of inducing or exacerbating IR [122].

To date, there has been limited investigation of the interaction of age and diet on glucose and insulin dynamics in healthy, non-obese horses. The objectives of this study were: 1) to measure minimal model parameters during an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT) after adaptation to a forage only diet or a forage diet supplemented with either a cereal grain based, starch and sugar (hydrolysable CHO) rich or an oil and fiber rich complementary concentrate feed; and 2) to assess glucose and insulin responses to a standardized hydrolysable CHO rich meal challenge (SMC) after diet adaptation in groups of healthy adult and aged horses. It was hypothesized that aged horses, as compared to adult horses, would have a greater acute insulin response to glucose administration (AIRg) and lower insulin sensitivity (SI) during the FSIGTT, regardless of diet. Similarly, glucose and insulin responses to the SMC were hypothesized to be greater in aged than adult horses, again regardless of diet. Finally, in both age groups adaptation to a cereal grain supplemented diet was hypothesized to produce a greater AIRg and a lower SI during the FSIGTT and greater glucose and insulin responses to the SMC, as compared to the other diets.

Materials and methods

Horses and housing

Seventeen mares, including eight adult (8.1 ± 1.6, range 5-10 yr) and nine aged (21.9 ± 1.6, range 19-24 yr) horses, were studied. Breeds included 12 stock-type horses, four Thoroughbreds, and one Standardbred. Aged mares weighed less than adult horses (455 ± 12 vs. 500 ± 13 kg, $P<0.02$) throughout the study but body condition score (BCS scale 1-9 [47]) was

not different between age groups (median [range] 5 [4-7] for adults and 5 [3-6] for aged, P=0.20). Prior to the study start, all horses underwent a complete oral examination to ensure presence of all premolars plus molars and minor dental abnormalities were corrected. All horses also received a dose of ivermectin paste. All aged mares had normal overnight dexamethasone suppression test results (ODST, cortisol suppression [<1.0 ug/dL] 19 h after dexamethasone administration, 40 µg/kg, IM [24]) and lacked clinical signs of pituitary pars intermedia dysfunction (PPID) [98]. Horses were grouped as pairs, one adult and one aged mare, and maintained in snow covered paddocks or dry lots to minimize pasture access (January-June).

Study design

Mares were studied in a Latin square design and fed three diets for 41 d: forage only (HAY); forage supplemented with a high starch and sugar (SS, rich in hydrolysable CHO) cereal grain based feed (Pleasure Sweet, Buckeye® Nutrition); or forage supplemented with a low starch and sugar, oil and fiber rich feed (FF, Equilibrium Growth, Winergy®). After 21 d on each diet, pairs were moved indoors and housed in adjacent stalls (ambient temperature, 15 ± 7°C) and fed individually for an additional 20 d. While housed in stalls, horses were turned out for exercise in a dry lot for 2 h, 3 d each week. The start of each diet period was staggered by either 2 or 4 wk due to the limited number of stalls available; consequently, testing was performed with groups of 5-6 mares at one time. Mares were initially fed 1.6% of body weight (BW), as fed, divided into two equal feedings (0700 and 1700 h): HAY = 1.6% BW hay; SS or FF = 1.0% BW hay and 0.6% BW SS or FF. On d 22 and d 41 of each period, horses were weighed and BCS was assessed by three trained individuals. By d 41 of the first period for the initial cohort of mares studied (February), BW had decreased (~16 and ~27 kg for aged and adult horses, respectively). Consequently, for the remainder of the study, the amount fed was increased

to 1.84% BW, as fed: HAY = 1.84% BW hay; SS or FF = 1.15% BW hay and 0.69% BW SS or FF.

Insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT)

An insulin-modified FSIGTT [49,109] was performed on d 31 or 32 of each feeding period (two or three horses each day). During the afternoon prior to testing, 13.3 cm, 14 G polyurethane catheters were aseptically inserted into both jugular veins and patency was maintained by injection of 1 mL of Na heparin (1000 IU/mL). Mares were fasted overnight (~12 h) prior to testing. Starting at 0900 h, two baseline blood samples were collected 15 min apart prior to glucose administration (0.1 g/kg, IV bolus, 50% dextrose solution, VEDCO, Inc.), followed 20 min later with insulin administration (20 mU/kg, IV bolus, Novolin R, Novo Nordisk, Inc.). Blood samples were collected from the opposite IV catheter at -15 ,-1, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min after glucose administration. At each sampling time, a 15 mL blood sample was collected with 4 mL transferred to a plastic tube containing lithium heparin, placed on ice and centrifuged (1,500g for 15 min at 5˚C) within 30 min of collection and 10 mL transferred to a tube without anticoagulant that was allowed to clot at room temperature for 2 h prior to centrifugation. Plasma and serum were harvested and stored at -80˚C until analysis.

Standardized meal challenge (SMC)

On d 42 of each feeding period, horses were fed a standardized amount of the cereal based meal (4 g/kg BW of SS) without hay for their morning feeding. A 13.3 cm, 14 G polyurethane catheter was aseptically inserted into the jugular vein during the afternoon prior to the SMC and patency of the catheter was maintained by injection of 1 mL of Na heparin (1000 IU/mL). Again, mares were fasted overnight (~12 h) prior to testing and three baseline blood

samples were collected during the 30 min prior to the SMC (offered at 0900 h) to determine baseline serum glucose and insulin concentrations. Feed remaining 1 h after offering the SMC was removed and weighed. Blood samples were collected 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, and 360 min after the meal was offered. At each time point, a 15 mL blood sample was collected and processed as described for the FSIGTT.

Due to the staggered start of diet period one, six FSIGTT and SMC tests were performed when mares were on the lower plane of nutrition (1.6% BW, two mares on each diet) while all other tests were performed after at least 1 week on the higher plane of nutrition (1.84% BW). The study protocol and all procedures performed were approved by the Michigan State University Institutional Animal Care and Use Committee (approval #11/09-174-00).

Sample analysis

Hay and concentrate feed samples were collected several times during the course of the study and proximate analysis of pooled, well-mixed samples was performed [28]. Additional aliquots of pooled feed samples were submitted for analysis of starch, water soluble CHO, and ethanol soluble CHO (Dairy One DHIA Forage Testing Laboratory). Plasma glucose concentrations were measured in duplicate using a membrane based glucose oxidase system to catalyse oxidation of glucose to gluconic acid and hydrogen peroxide (YSI 2300 STAT Plus Glucose and Lactate Analyzer, Yellow Springs Instruments). Serum insulin concentrations were determined in duplicate using a radioimmunoassay (Coat-a-Count Insulin, Siemens Medical Solutions Diagnostics) previously validated for equine samples [108]. Inter-assay CV was 9.3% and intra-assay CVs were 5.2% and 8.6% for high and low control samples, respectively.

Calculations and statistical analysis

SI, AIRg, glucose effectiveness (Sg) and disposition index (DI = SI × AIRg) were calculated by minimal model analysis of glucose and insulin data from the FSIGTT (MinMod Millenium, Version 6.02; [10]). For the SMC, peak glucose and insulin concentrations and time to peak concentrations were determined, and area under the 360 min curves for glucose (AUCg) and insulin (AUCi) were calculated by the trapezoidal method, using commercially available computer software (GraphPad Prism, version 6.0, GraphPad Software Inc., San Diego, CA). Outlier data points were identified by use of the Grubbs test, with a critical value of $P \leq 0.05$. One outlier (adult) for the FF diet was removed from analysis of insulin response during the SMC. Differences between ages and diets for minimal model variables and SMC were determined via mixed ANOVA for repeated measures and Tukey-Kramer post hoc adjustment (SAS, version 9.4, SAS Institute Inc., Cary, NC). Data in the text are presented as means ± SD and significance was set at $P \leq 0.05$.

Results

Nutrient composition of the three feedstuffs is presented in Table 1. The SS concentrate contained 43% NSC, ~3 and 4 times more NSC than FF and HAY, respectively, while the FF feed contained 11% fat, ~1.5 and 2.3 times more fat than SS and HAY, respectively. When either supplemental feed was combined with hay, digestible energy provided was ~12% (FF) to ~19% (SS) greater when compared to HAY alone: 40.6 ± 0.9, 45.6 ± 0.7, and 48.3 ± 0.9 kcal/kg/d for HAY, FF and SS, respectively, ($P<0.01$, with SS and FF significantly greater than HAY). Overall, horses consumed the diets well over the course of the study. As mentioned, BW decreased ($P<0.05$) in both age groups from barn entry on d 22 to barn exit on d 41 during period 1, regardless of diet (Table 2). BW loss was attributed to the combined effects of season (winter) and a transient period of decreased feed intake when horses were initially placed into stalls

during period 1, despite having been previously housed in the same facility. Once the amount of feed provided was increased, BW and BCS tended to increase a small amount over the remainder of the study in both adult and aged mares (Table 2).

Minimal model parameters

AIRg was significantly affected by age ($P<0.02$), with mean AIRg greater in aged when compared to adult mares across all diets. In aged mares AIRg was lower ($P<0.05$) after adaptation to the HAY diet when compared to SS; AIRg did not differ among diets in adult mares. SI was significantly greater ($P<0.01$) in adult when compared to aged mares, regardless of diet (Table 3). Further, a significant age × diet interaction ($P<0.01$) was also detected. For adult mares there was no difference in SI among the three diets but in aged mares SI was lower with HAY and FF when compared to SS (Table 3). Although there was no difference ($P=0.08$) between age groups, DI was greater ($P<0.01$) after adaptation to the SS diet, as compared to the HAY diet. In adult mares, mean DI was also greater ($P<0.01$) with FF than HAY. There were no significant differences in Sg with either age or diet (Table 3).

Insulin and glucose dynamics during the SMC

Glucose (Fig. 1) and insulin (Fig. 2) concentrations reached peak values within 90-120 min after the meal was offered. There was no effect of age ($P=0.32$) or diet ($P=0.10$) on peak glucose concentration after eating a meal rich in hydrolysable CHO. Similarly, time to peak glucose concentration was not affected by age ($P=0.13$) or diet ($P=0.10$). In contrast, peak insulin concentration, but not time to peak concentration, was greater ($P<0.03$) in aged when compared to adult mares. There was no effect of diet on peak ($P=0.80$) or time to peak ($P=0.18$) insulin concentration (Table 4).

AUCg was not different (P=0.66) between age groups, regardless of diet (Table 4). However, AUCg was lower (P<0.05) after adaptation to SS, in comparison to HAY, in both age groups. AUCi was lower (P<0.03) in adult mares, as compared to aged mares, regardless of diet (Table 4). Although AUCi in adult horses was 40% lower after adaptation to SS, as compared to the other diets fed to this age group, this was not a significant finding (P=0.29). Not surprisingly, AUCg and AUCi were positively correlated (r=0.44, P<0.01).

A limitation of the SMC was incomplete ingestion, defined as leaving more than 0.4 kg (~20%) of the meal after 60 min, by four mares (two adult and two aged mares) in eight of the trials (one, four, and two of these instances occurring after adaptation to HAY, FF, and SS, respectively). When data for these eight studies were compared to data for the remaining 36 studies during which >90% of the meal was ingested, AUCg was lower (P<0.03) for the eight studies with partial meal consumption but there was no difference in AUCi (P=0.78) (Fig. 3).

Discussion

In the study reported here, glucose and insulin dynamics were affected by age and diet in healthy horses. Consistent with findings of Liburt et al. [64], AIRg was greater and SI was lower in aged mares when compared to adult mares. Notably, diet did not significantly affect AIRg or SI in adult mares; whereas, both SI and AIRg were greater in aged mares after adaptation to SS when compared to HAY (and FF for SI). These age- and diet-associated differences in glucose and insulin dynamics in response to IV glucose challenge were, for the most part, corroborated by findings during the SMC. Specifically, peak insulin concentration and AUCi were greater in aged than in adult mares with all diets. In contrast to our hypothesis, however, AUCg in aged mares was lower after adaptation to SS when compared to the other diets, although this finding was consistent with the greater SI observed in aged mares adapted to this diet.

A greater increase in circulating insulin concentration, in response to both IV glucose (AIRg) and ingestion of the SMC, in aged mares could be a consequence of increased pancreatic insulin release, decreased insulin clearance, or a combination of the two. A growing body of evidence in other species suggests that insulin action on target tissues decreases with age, due to decreased responsiveness of insulin signaling pathways that lead to translocation of GLUT-4 vesicles to the plasma membrane [12, 43, 79, 123]. Multiple factors including increased visceral adiposity, decreased lean muscle mass, mitochondrial dysfunction, inflammation and oxidative stress, and reduced physical activity all contribute to development of IR with ageing in people [20]. As a consequence, a greater pancreatic β-cell insulin release is required to re-establish euglycemia following a meal. Similar to people, aged horses tend to have decreased muscle mass (supported by a lower BW despite similar size in aged mares in this study) and less structured physical activity and these factors likely contribute to aging-associated IR in this species as well. In support, exercise training of aged mares has been shown to decrease AIRg and improve SI, although the former was not a significant finding [64].

Cellular mechanisms of IR with aging in horses have been little studied. Waller et al. [121] demonstrated decreased basal and insulin-stimulated skeletal muscle cell surface GLUT-4 expression in IR mares, as compared to insulin sensitive mares, despite similar skeletal muscle cell GLUT-4 content. This finding suggests that insulin signaling pathways are dysfunctional in IR horses, unrelated to age. Increased amounts of inflammatory cytokines (tumor necrosis factor-α, interleukin-1, and interleukin-6) have also been measured in blood and insulin-sensitive tissues in IR horses, although studies have yielded conflicting results and age may have confounded the results [105, 117, 120]. A possible role for inflammatory cytokines and oxidative stress, implicated as contributing factors to mitochondrial dysfunction with age in people [20], in

development of IR with aging in horses remains unclear [64]. Finally, although all aged mares had normal ODST results, this test may be insensitive for detection of earlier stages of PPID ([76]; D. McFarlane, personal communication 2015). Thus, differences in function of the hypothalamic-pituitary-adrenal axis between adult and aged mares cannot be excluded as an additional factor contributing to development of IR with aging in horses.

Insulin is largely cleared (more than 50% of β-cell release) from portal blood during first pass through the liver and the remainder is cleared by uptake and degradation in target tissues and renal elimination [21]. A potential role for altered hepatic insulin clearance in development of IR with aging in horses remains uncertain. An early study found a longer half-life of exogenous insulin in horses (33 min) as compared to people (3-8 min) [67], suggesting that peripheral insulin clearance can also differ between species. Finally, a decline in renal function in aged animals could prolong insulin half-life; however, decreased hepatic or renal elimination would be an unlikely explanation for either the immediate insulin response during the FSIGTT (AIRg) or postprandially following the SMC. These acute insulin responses suggest that increased pancreatic β-cell secretion plays a greater role, as compared to alterations in clearance, in the exaggerated insulin response observed in aged mares.

With regard to diet, our hypothesis that AIRg would be greater after adaptation to SS, when compared to HAY or FF, was only supported in aged horses (AIRg was numerically highest in adult horses after adaptation to SS, but this was not a significant finding). In contrast, our hypothesis that SI would be lower after adaptation to SS, as compared to HAY or FF, was refuted. In fact, SI was greatest in aged horses after adaptation to SS (again, although not a significant finding, SI was also numerically highest after adaptation to SS in adult horses). A similar finding of a greater SI (but not a greater AIRg) after 20 wk of feeding a glucose

supplemented (1.5 g/kg BW of glucose) meal once daily to increase adiposity in horses and ponies (aged 5-19 years) was recently reported by Bamford et al. [7]. This finding also contradicted their hypothesis that inducing obesity by increasing NSC intake would decrease SI, as had been previously reported in Arabian horses that were fed increasing amounts of a sweet feed to induce obesity [11]. They suggested that obesity *per* se may not affect insulin sensitivity, at least over a short period of time, while chronic obesity may pose a different risk. Similar to our findings, when obesity was induced by feeding an oil supplemented diet (25% of DE as oil), SI was unchanged [7]. Disposition index, the product of AIRg and SI, was nearly 3-fold greater in both age groups after adaptation to SS, supporting greater β-cell responsiveness to IV glucose administration [16] after adaptation to a diet high in hydrolysable CHO. In contrast, the ability of glucose to drive its own disposal (tissue uptake) without the influence of insulin (Sg) was not different between diets or age groups. This finding suggests that neither diet nor age had a significant effect on peripheral tissue glucose transporters that are not regulated by insulin (i.e., GLUT-1 and GLUT-12) [2, 119].

The finding of a lower AUCg response to the SMC after adaptation to SS in both age groups was also contrary to our hypothesis. However, adaptation to SS likely increased small intestinal capacity for glucose uptake by upregulation of sodium/glucose cotransporters (SGLT1) on enterocytes [14, 102]. SGLT1 expression is regulated by the enteric nervous system. Increased amounts of luminal sugars lead to greater secretion of gut hormones (incretins), via stimulation of the sweet receptor expressed on enteroendocrine cells that are dispersed within the intestinal mucosa [102]. These hormones include glucagon-like peptides 1 and 2 (GLP-1 and GLP-2) and glucose-dependent insulinotrophic peptide (GIP). Intravenous administration of GLP-2 increases SGLT1 expression in absorptive enterocytes indirectly, through stimulation of

GLP-2 receptors expressed on enteric neurons [17]. In people, release of incretins GLP-1 and GIP during a meal accounts for 50–60% of pancreatic insulin secretion by stimulating specific receptors on pancreatic β-cells [81]. Both upregulation of SGLT1 transporters and increased activity of the enteroinsular axis after adaptation to SS should increase the rate of glucose absorption and uptake by peripheral tissues. Thus, the lower AUCg response to the SMC after adaptation to SS in both age groups could potentially be explained by increased intestinal glucose uptake in combination with enhanced hepatic and peripheral tissue glucose disposition, blunting the rise in circulating glucose concentration.

There were several limitations of this study including transient weight loss in the first cohort of horses studied in period 1; however, this problem occurred with both age groups and across diets. It should be emphasized that the SMC was designed to be an integrated assessment of intestinal CHO absorption, the enteroinsular axis, and tissue glucose disposition, rather than an attempt to develop a diagnostic test for IR. Incomplete meal ingestion would represent a substantial limitation as a diagnostic test, as compared to oral sugar administration [99]. Fortunately, in this study incomplete ingestion of the SMC was similar across age groups and diets, minimizing any confounding effect.

Conclusions

Insulin responses to IV or enteral CHO challenge increase with age in healthy horses, regardless of diet fed. As people reach advanced age, β-cell senescence appears to be an important factor for eventual β-cell failure (and development of uncompensated IR and type 2 diabetes mellitus) [20]. Our group of aged horses studied was not overweight and did not have evidence of β-cell failure or PPID. It warrants comment that the definition of "aged" remains to be established for horses, as many horses between 20-30 years of age remain healthy and in

regular exercise. Curiously, despite a greater SI and a modest postprandial increase in insulin response, in response to a high hydrolysable CHO meal challenge, after adaptation to the SS diet in aged mares, glucose clearance (AUCg) improved in both age groups after adaptation to the SS. This finding could suggest that addition of some hydrolysable CHO to a forage diet may actually sensitize the enteroinsular axis and enhance postprandial glucose clearance, regardless of age. However, further studies are needed before such a dietary recommendation could be made, especially for ponies and other breeds with lower insulin sensitivity as well as for overweight equids. Finally, it remains unclear whether or not intermittent postprandial hyperinsulinemia, and possibly subclinical PPID, may place older horses at greater risk for development of laminitis.

CHAPTER 3

CONCLUSIONS

This study was the first to look at the effects of age and diet on tissue sensitivity to insulin in adult and aged horses. Consistent with previous reports [64, 69], older horses have reduced tissue sensitivity to insulin. Limited research has been conducted looking at the underlying mechanism. The greater circulating insulin concentrations in aged horses could be a result of increased pancreatic output, decreased insulin clearance or a combination of both. The insulin pathway could have fewer insulin receptors available on the cell surface, decreased receptor affinity to insulin, or some downstream pathway defect. There is evidence that tissue sensitivity to insulin decreases with age due to a decrease in the translocation of GLUT-4 vesicles to the plasma membrane [12, 43, 79, 123].

In human literature, it is widely accepted that increased adipose tissue, decreased lean muscle mass, mitochondrial dysfunction, inflammation and oxidative stress, and reduced physical activity all contribute to the development of insulin resistance [20]. Aged horses have been reported to be in a pro-inflammatory state which could lead to insulin resistance. Two pro-inflammatory cytokines, TNF-alpha and IL-1beta, are thought to impair insulin sensitivity whereas IL-6 appears to improve insulin sensitivity by down regulating TNF-alpha and IL-1 beta. Increased amounts of inflammatory cytokines (TNF-alpha, IL-1, and IL-6) have been measured in blood and tissues of insulin resistance horses [105, 117, 119]. The role of these inflammatory cytokines and oxidative stress on mitochondrial dysfunction in the development of insulin resistance remains unclear however.

In addition to age, diet is an important consideration when examining glucose and insulin dynamics. When comparing the three diets (HAY, SS, and FF), horses adapted to a HAY diet

had a significantly greater glycemic response. This finding is supported by Murphy and colleagues who reported a greater area under the curve for glucose when adapted to hay only diet as compared to a high fiber pelleted meal [80]. Interestingly, we also found greater insulin sensitivity when mares were adapted to the SS diet which did not support our hypothesis or previous research [90] which reported a greater area under the curve for insulin following a high sugar and starch diet. Pratt et al. also reported an increase in body weight and body condition score which may have influenced their results. Additional research reported lower insulin sensitivity when adapted to the high starch and sugar diet as compared to a fat and fiber diet [49]. One limitation of this paper is only 4 horses were studied so sample size could be a confounding variable for the results.

Like the effect of age on insulin sensitivity, limited research has been done to identify the mechanism behind diet adaptation and the effect on insulin sensitivity. There are a variety of possible mechanisms leading to improved glucose uptake following diet adaptation to the high sugar and starch meal. Diet adaptation may increase the number of insulin receptors on the cell surface, increase the affinity of the insulin receptor, or up regulate downstream signaling within the insulin pathway leading to an increased number of GLUT-4 receptors for glucose uptake. These are just a couple of the many possible causes for why mares adapted to a starch and sugar feed showed improved insulin sensitivity. To further explore mechanisms, a review of how glucose is absorbed by the gastrointestinal tract is warranted.

Many horses today have been adapted to a diet of forage and concentrate. The concentrate is typically added to provide enough energy for the demands of work and performance. These concentrates are high in hydrolysable carbohydrates which are broken down in the small intestines by pancreatic alpha-amylase and the brush border disaccharidases to

monosaccharides such as glucose. Glucose and galactose are transported across the brush border membrane of the enterocytes by SGLT1/Na+/glucose transporter while fructose is transported by a sodium independent transporter, GLUT5. These monosaccarides accumulate in the enterocytes until moving down a concentration gradient on the basolateral side by GLUT2 [9]. Horses adapted to a pasture diet have the greatest glucose absorption in the proximal intestine (duodenum>jejunum) with very low expression of SGLT1 in the ileum [25].

Although limited research has been conducted looking at diet adaptation in the small intestines of the horse, Dyer and colleagues studied the expression of SGLT1 within the duodenum and ileum of 6 adult Standardbred geldings. Following 3 months of adaptation to a timothy hay diet, biopsies of the duodenum and ileum were harvested via laparoscopic technique. Horses were then introduced to 60 % hay, 40 % grain (3.3 g starch/kg bwt/day) diet. Biopsies were taken after 1 week and 1 month. Lastly, horses were adapted to 40 % hay, 60 % grain (6.0 g starch/kg bwt/day) and biopsies were harvested after 1 month. When adapted to the hay only diet, the greatest abundance of SGLT1 receptors was in the duodenum as compared to the ileum. After 1 week of eating the concentrate, there was no change in the duodenum expression but a 2 fold increase in SGLT1 expression in the ileum. After 1 month adaptation to the diet, the ileum expression did not change from 1 week of adaptation but the duodenum expression doubled. Lastly, after increasing the amount of concentrate for 1 additional month, no further expression was appreciated in the duodenum but the ileum had a 3 fold higher expression than the hay only diet. A similar pattern was seen with the GLUT2 transporter suggesting that adaptation to the high sugar and starch diet increased glucose transport into the blood stream by the enterocytes [26]. More recent work supports the upregulation of the SGLT1 on enterocytes following adaptation to a high sugar and starch meal [17, 102].

The amount of insulin released to accelerate glucose uptake is partly regulated by the enteroinsular axis which consists of both neural and hormonal factors. When the small intestine lumenal glucose is above threshold, the sweet receptor is activated. This receptor causes secretion of gastrointestinal hormones called incretins. Incretins are synthesized by the endocrine cells of the gastrointestinal tract and are released with food absorption. Incretins promote release of insulin under hyperglycemic conditions. Insulin secretion following an oral glucose tolerance test is greater than an intravenous bolus of glucose because incretins are activated [22]. In humans, there are two gut hormones shown to act on incretins, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide (GLP-1). Another hormone, GLP-2 does not increase insulin secretion but does modulate intestinal growth, blood flow, and SGLT1 in several species. The receptors for GLP-2 are in the enteric neurons suggesting the enteric nervous system is involved in upregulation of SGLT1 receptors as well. It has been shown that GIP, GLP-1,GLP-2 and sweet receptors are in the horse intestines [6,18]. GLP-1 concentration was positively correlated with insulin concentration postprandially documenting an association between incretins and insulin concentration in equids [6].

Duhlmeier and colleagues investigated the enteroinsular axis in Shetland ponies and Standardbred horses following an oral glucose tolerance test and intravenous glucose tolerance test. Results showed an increased GIP following the oral glucose challenge only. One pony had an exaggerated response to both of the challenges indicating insulin resistance. This pony had a 3 times higher GIP level than the other ponies and horses in the study supporting a greater insulin secretion requirement to uptake glucose for an insulin resistant animal [22]. A similar study investigated the effect of different diets on the enteroinsular axis of 8 Shetland ponies. Ponies fed a high fat diet for 5 weeks had a greater plasma GIP concentration that those adapted

to a sugar and starch diet. This increase in GIP may be a stimulus for insulin hypersecretion and insulin resistance [97].

Glucose uptake from the blood stream by the skeletal muscle and adipose tissue is stimulated by increased insulin concentration via the insulin receptor on the cell surface. The main receptor that facilitates glucose uptake is GLUT4 and partially by GLUT12 [104]. When there is an increase in insulin concentration, translocation of intracellular GLUT4 to the cell surface occurs resulting in glucose uptake. This is the rate limiting step in glucose uptake in insulin sensitive tissues. Waller and colleagues assessed the GLUT4 and GLUT12 expression in both muscle and adipose tissue of 10 light breed mares, 5 of which were considered insulin resistant based on FSIGT results. They found that insulin resistance selectively decreased active basal cell surface GLUT4 in skeletal muscle without altering GLUT12 or total GLUT4 or GLUT 12 content. Furthermore, there was no enhancement with in vitro insulin stimulation. These insulin resistant mares showed diminished expression of GLUT4 in omental fat which had the greatest GLUT4 content in insulin sensitive mares [120, 121]. These results suggest that the mares in our study could have had an upregulation of GLUT4 transporters to the cell surface following diet adaptation. This would allow for increased glucose uptake following a glucose bolus when adapted to the high sugar and starch diet.

Our research further supports an age-related increase in insulin resistance in healthy, non-obese horses. Diet is an important consideration when studying glucose and insulin dynamics. We reported increased tissue sensitivity to insulin following adaptation to a high sugar and starch diet. This finding raises a variety of management concerns. For example, would a horse with the phenotype of a Metabolic Syndrome horse benefit from introducing small amounts of a high sugar and starch diet prior to being placed on lush, spring pasture. The amount of concentrate

and the duration needed to see the improved insulin sensitivity was beyond the scope of this study but would be one area where further investigation is warranted. The exact mechanisms behind how age and diet affect tissue sensitivity to insulin remain unknown but are interesting areas for future research.

APPENDIX

Table 1 Nutrient composition of the three diets fed, expressed on a dry matter basis, determined by proximate analysis of well-mixed composite samples of several aliquots of each feedstuff collected multiple times during the study; digestible energy (DE) of each diet was calculated using measured DE for each food stuff and the amount fed: HAY = grass hay; FF = mix of hay and fat and fiber rich concentrate feed; and SS = mix of hay and cereal grain feed rich in nonstructural CHO (sugar and starch feed).

	HAY	**SS**	**FF**
NDF (%)	61.4	25.0	42.3
CP (%)	7.9	13.2	14.9
Fat (%)	3.56	5.28	8.30
Calcium (g/kg)	7.9	11.4	15.9
Phosphorus (g/kg)	1.6	7.5	5.4
Lignin (%)	6.9	2.8	4.1
WSC (%)	10.6	7.6	8.6
ESC (%)	5.7	7.0	6.9
Starch (%)	0.5	35.2	5.4

Table 2 Bodyweights (BW) and body condition scores (BCS) at barn entry (day 22) and at the end (day 41) of each feeding period in eight adult horses and nine aged horses. Values reported as means ± SEM.

		Period 1		Period 2		Period 3	
	Group	day 22	day 41	day 22	day 41	day 22	day 41
BW (kg)	adult*	509 ± 10^{a}	482 ± 12^{x}	517 ± 12^{a}	522 ± 12^{y}	518 ± 11^{a}	520 ± 7^{y}
	aged	466 ± 13^{ab}	449 ± 13^{x}	456 ± 15^{a}	453 ± 17^{x}	464 ± 15^{b}	464 ± 16^{y}
BCS (1-9)	adult	5.1 ± 0.1^{a}	4.9 ± 0.2^{x}	5.2 ± 0.1^{ab}	5.3 ± 0.1^{xy}	5.4 ± 0.2^{b}	5.6 ± 0.2^{y}
	aged	4.8 ± 0.3^{ab}	4.6 ± 0.2^{x}	4.5 ± 0.3^{a}	4.7 ± 0.3^{x}	5.0 ± 0.3^{b}	5.1 ± 0.3^{x}

* indicates significant ($p<0.05$) difference between adult and aged horses for all times
Within age group, means with different superscript letters differ at $P < 0.05$ (a,b for day 22 [barn entry] and x,y for day 41 [period end])

Table 3 Acute insulin response to glucose (AIRg) infusion, insulin sensitivity (SI), glucose effectiveness (Sg), and disposition index (DI) determined by minimal model analysis of glucose and insulin data obtained from an insulin-modified frequently sampled i.v. glucose tolerance test performed in eight adult horses and nine aged horses that had been adapted to three diets for 4 wk: HAY (grass hay only); SS (hay plus a cereal grain feed rich in hydrolyzable CHO [sugar and starch feed]); and FF (hay plus a fat and fiber rich concentrate feed). Values reported as means ± SEM.

	Group	HAY	SS	FF
AIRg ([mU/•L^{-1}]•min)	adult*	113.1 ± 20.4[a]	157.9 ± 24.7[a]	140.4 ± 19.2[a]
	aged	209.7 ± 29.5[a]	286.0 ± 35.5[b]	238.1 ± 28.5[ab]
SI (L•min^{-1}•mU^{-1})•10^{-4}	adult*	2.52 ± 0.53[a]	3.64 ± 0.55[a]	3.39 ± 0.52[a]
	aged	0.77 ± 0.27[a]	2.19 ± 0.35[b]	0.99 ± 0.24[a]
Sg (min^{-1}•10^{-2})	adult	1.87 ± 0.25	2.73 ± 0.32	2.34 ± 0.27
	aged	1.81 ± 0.23	1.90 ± 0.17	1.80 ± 0.21
DI (•10^{2})	adult	2.41 ± 0.76[a]	6.22 ± 1.1[b]	5.11 ± 0.78[b]
	aged	1.56 ± 0.65[a]	5.13 ± 0.89[b]	2.45 ± 0.67[a]

* indicates significant ($p<0.05$) difference between adult and aged horses for all dietary treatments

Within age group, means with different superscript letters differ at $P < 0.05$

Table 4 Measures of glucose and insulin responses before, during, and after the standardized CHO-rich meal challenge (4.4 g/kg of the SS cereal grain feed) administered in eight adult horses and nine aged horses that were adapted to three diets for 6 wk: HAY (grass hay only); SS (hay plus a cereal grain feed rich in nonstructural CHO [sugar and starch feed]); and FF (hay plus a fat and fiber rich concentrate feed). Values reported as mean ± SEM.

	Group	HAY	SS	FF
basal glucose ($mg \cdot L^{-1}$)	adult	90.9 ± 2.7	90.6 ± 1.7	90.3 ± 2.4
	aged	87.3 ± 3.0	89.5 ± 1.9	88.4 ± 2.7
peak glucose ($mg \cdot L^{-1}$)	adult	139.2 ± 6.5	124.2 ± 4.8	144.8 ± 9.3
	aged	142.9 ± 8.1	123.5 ± 4.4	134.3 ± 5.9
time to peak glucose (min)	adult	122.5 ± 12.0	105.0 ± 4.6	108.8 ± 6.8
	aged	136.9 ± 9.8	106.9 ± 7.3	118.3 ± 7.1
AUCg ($[mg \cdot L^{-1}] \cdot min) \cdot 10^3$	adult	88.0 ± 15.9[a]	49.1 ± 12.0[b]	81.2 ± 16.0[a]
	aged	112.8 ± 15.3[a]	52.8 ± 8.5[b]	79.2 ± 10.1[ab]
basal insulin ($mU \cdot L^{-1}$)	adult	2.7 ± 0.2	2.8 ± 0.6	3.3 ± 0.3
	aged	4.6 ± 1.0	4.9 ± 0.8	4.0 ± 0.3
peak insulin ($mU \cdot L^{-1}$)	adult*	64.5 ± 11.0	53.4 ± 7.9	65.2 ± 8.5
	aged	117.8 ± 16.4	151.6 ± 25.6	144.2 ± 19.9
time to peak insulin (min)	adult	120.0 ± 14.8	97.5 ± 3.7	113.6 ± 12.5
	aged	185.6 ± 36.1	125.6 ± 17.7	143.3 ± 18.4
AUCi ($[mU \cdot L^{-1}] \cdot min) \cdot 10^3$	adult*	12.9 ± 2.7	8.1 ± 1.3	12.2 ± 2.2
	aged	24.1 ± 3.0	22.6 ± 5.6	26.4 ± 3.9

* indicates significant ($p<0.05$) difference between adult and aged horses for all dietary treatments

Within age group, means with different superscript letters differ at $P < 0.05$

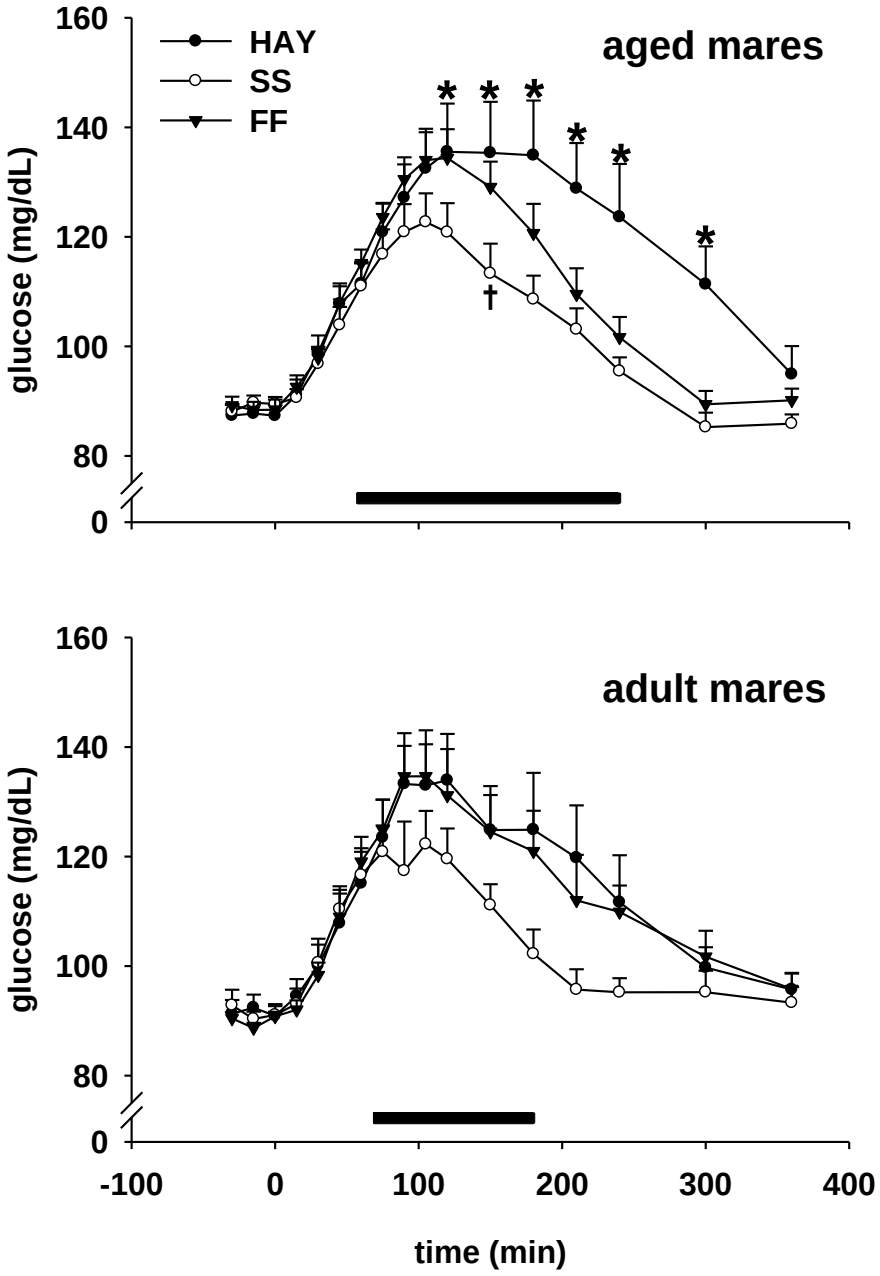

Figure 1 Mean ± SEM glucose concentrations in response to the standardized meal challenge (0.4 g/kg SS concentrate feed offered for 60 min) in adult (bottom panel) and aged (upper panel) mares. The filled bars above the x-axes indicate time points that were different (P <0.05) from baseline; asterisks indicate time points at which SS < HAY (P < 0.05); cross indicates a time point at which SS < FF (P < 0.05).

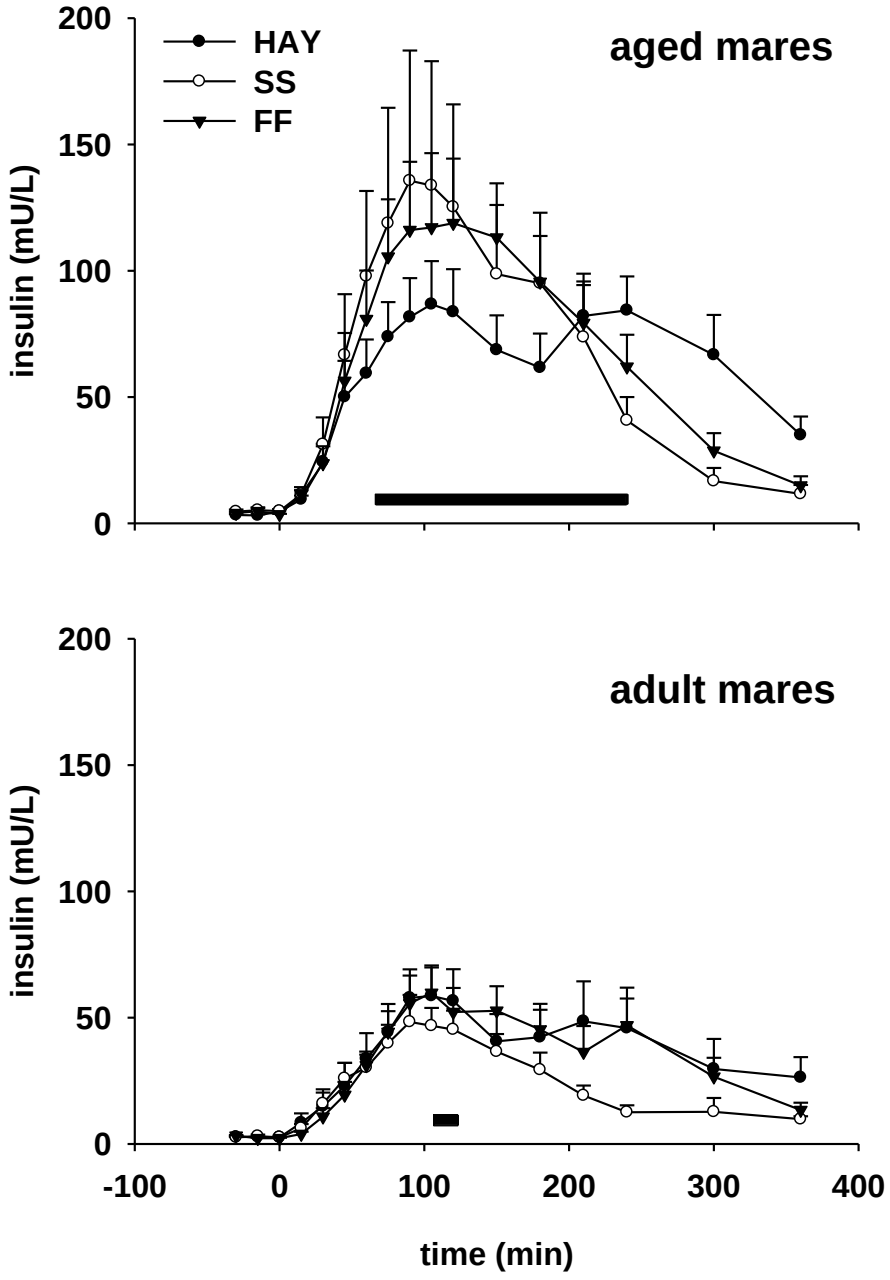

Figure 2 Mean ± SEM insulin concentrations in response to the standardized meal challenge (0.4 g/kg SS concentrate feed offered for 60 min) in adult (bottom panel) and aged (upper panel) mares. The filled bars above the x-axes indicate time points that were different (P <0.05) from baseline; asterisk indicates a time point at which SS > HAY (P < 0.05).

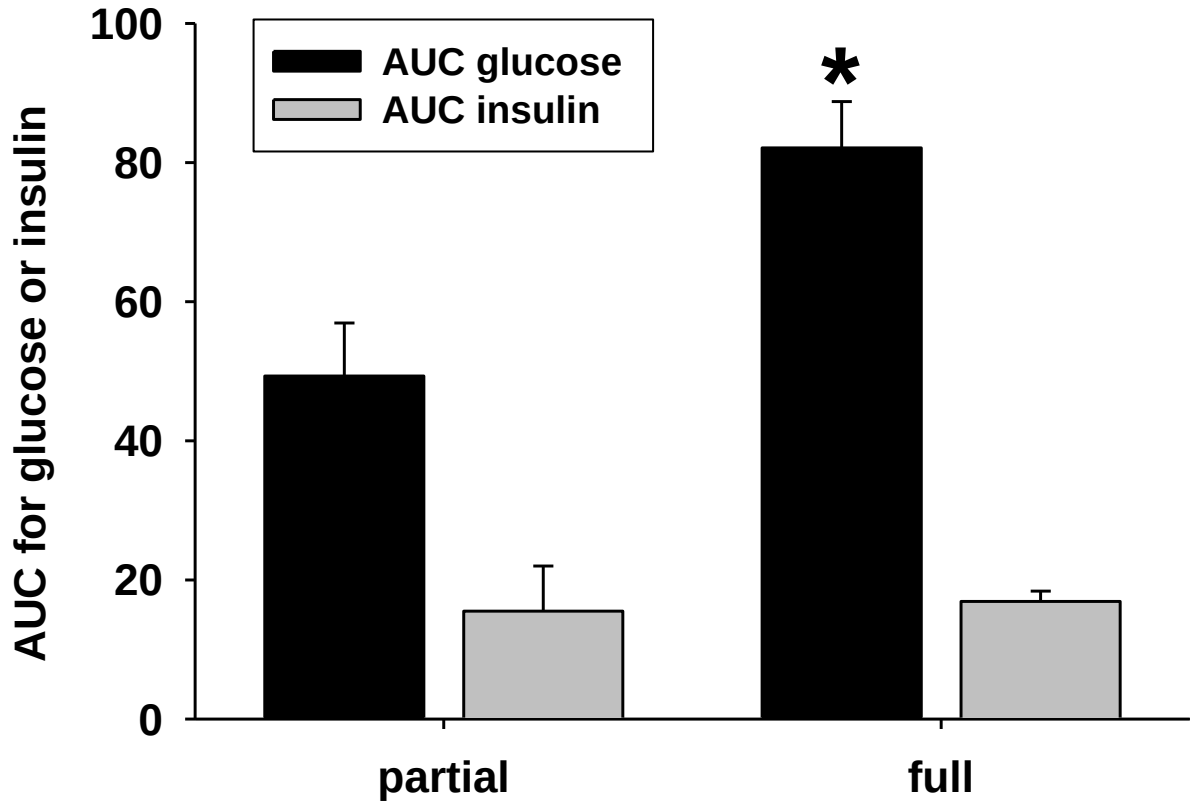

Figure 3 Mean ± SEM area under the curves for glucose (AUCg ([mg•L^{-1}]•min)•10^3, black fill) and insulin (AUCi ([mU•L^{-1}]•min)•10^3, gray fill) in mares that consumed >90% (full) of the SS meal in the standardized meal challenge (n = 36 studies) as compared to mares that left more the 20% (partial) of the SS meal after 60 min (* denotes $P < 0.05$).

REFERENCES

REFERENCES

1. Alberti, K. G. and P. Z. Zimmet, Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med,1998. **15**(7): p. 539-553.

2. Annandale, E. J., S. J. Valberg, et al., Insulin sensitivity and skeletal muscle glucose transport in horses with equine polysaccharide storage myopathy. Neuromuscul Disord, 2004. **14**(10): p.666-674.

3. Argenzio, R.A. and H.F. Hintz, Effect of diet on glucose entry and oxidation rates in ponies, J Nutr, 1972. **102**(7): p. 879-892.

4. Asplin, K. E., M. N. Sillence, et al., Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies, Vet J, 2007. **174**(3): p. 530-535.

5. Bamford, N. J., S. J. Potter, et al., Breed differences in insulin sensitivity and insulinemic responses to oral glucose in horses and ponies of moderate body condition score, Domest Anim Endocrinol , 2014. **47**: p. 101-107.

6. Bamford, N.J.,C.L. Baskerville, et al., Postprandial glucose, insulin, and glucagon-like peptide-1 responses of different equine breed adapted to meals containing micronized maize, J Anim Sci, 2015a. in press. Doi:10.2527/jas.2014-8736.

7. Bamford, N.J., S.J. Potter, et al., Effect of increased adiposity on insulin sensitivity and adipokine concentrations in horses and poines fed a high fat diet, with or without a once daily high glycaemic meal, Equine Vet J, 2015b. in press. Doi:10.1111/evj.12434.

8. Boden, G., K. Sargrad, et al., Effect of a low-carbohydrate diet on appetite, blood glucose levels, and insulin resistance in obese patients with type 2 diabetes, Ann Intern Med, 2005. 143(6): p. 403-411.

9. Boron, W.F. and E.L. Boulpaep, Medical Physiology, 2009. Philadelphia, PA: Saunders.

10. Boston, R. C., D. Stefanovski, et al., MINMOD Millennium: a computer program to calculate glucose effectiveness and insulin sensitivity from the frequently sampled intravenous glucose tolerance test, Diabetes Technol Ther, 2003. **5**(6): p. 1003-1015.

11. Carter, R. A., L. J. McCutcheon, et al., Effects of diet-induced weight gain on insulin sensitivity and plasma hormone and lipid concentrations in horses, Am J Vet Res, 2009.**70**(10): p.1250-1258.

12. Couet, C., J. Delarue, et al., Age-related insulin resistance: a review, Horm Res,1992. **38**(1-2): p. 46-50.

13. Cunningham, J.G. and B.G. Klein, Veterinary Physiology. 2007. St. Louis, MO: Saunders

14. Daly, M. E., C. Vale, et al., Acute effects on insulin sensitivity and diurnal metabolic profiles of a high-sucrose compared with a high-starch diet, Am J Clin Nutr,1998. **67**(6): p.1186-1196.

15. Dashti, H.M., T.C. Matthew, et al., Beneficial effects of ketogenic diet in obese diabetic subjects, Mol Cell Biochem, 2007. **302**(1-2): p. 249-256.

16. Denti, P., G. M. Toffolo, et al., The disposition index: from individual to population approach, Am J Physiol Endocrinol Metab, 2012. **303**(5): p. E576-586.

17. Daly, K., M. Al-Rammahi, et al., Expression of sweet receptor components in equine small intestine: relevance to intestinal glucose transport, Am J Physiol Regul Integr Comp Physiol, 2012. **303**(2): p. R199-208.

18. de Graaf-Roelfsema, E.,Glucose homeostasis and the enteroinsular axis in the horse: A possible role in equine metabolic syndrome,Vet J, 2014. **199**: p. 11-18.

19. de Laat, M. A., C. M. McGowan, et al., Hyperinsulinemic laminitis, Vet Clin North Am Equine Pract, 2012. **26**(2): p. 257-264.

20. de Tata, V., Age-related impairment of pancreatic beta-cell function: pathophysiological and cellular mechanism, Front Endocrinol, 2014. **5**: article 138.

21. Duckworth, W. C., R. G. Bennett, et al., Insulin degradation: progress and potential, Endocr Rev, 1998. **19**(5): p. 608-624.

22. Duhlmeier, R., E. Deegen, et al., Glucose-dependent insulinotropic polypeptide (GIP) and the enteroinsular axis in equine (Equus caballus), Comp Biochem Physiol A Mol Integr Physiol, 2001. 129(2-3): p.563-575.

23. Durham, A. E., K. J. Hughes, et al., Type 2 diabetes mellitus with pancreatic beta cell dysfunction in 3 horses confirmed with minimal model analysis, Equine Vet J, 2009. **41**(9): p. 924-929.

24. Dybdal, N. O., K. M. Hargreaves, et al., Diagnostic testing for pituitary pars intermedia dysfunction in horses, J Am Vet Med Assoc, 1994. **204**(4): p. 627-632.

25. Dyer, J., E. Fernandez-Castano Merediz, et al., Molecular characterization of carbohydrate digestion and absorption in equine small intestine, Equine Vet J, 2002. **34**(4): p. 349-358.

26. Dyer, J., M. Al-Rammahi, et al., Adaptive response of equine intestinal Na+/glucose co-transporter (SGLT1) to an increase in dietary soluble carbohydrate, Pflugers Arch, 2009. **458**(2): p. 419-430.

27. Eiler, H., N. Frank, et al., Physiologic assessment of blood glucose homeostasis via combined intravenous glucose and insulin testing in horses, Am J Vet Res, 2005. **66**(9): p. 1598-1604.

28. Elzinga, S., Nielsen, B.D., et al., Comparison of nutrient digestibility between adult and aged horses, J Equine Vet Sci, 2014. **34**(10): p. 1164-1169.

29. Ferrannini, E. and A. Mari, How to measure insulin sensitivity, J Hypertens, 1998. **16**(7): p. 895-906.

30. Fink, R. I., P. Wallace, et al., Effects of aging on glucose-mediated glucose disposal and glucose transport, J Clin Invest, 1986. **77**(6): p. 2034-2041.

31. Firshman, A. M. and S. J. Valberg, Factors affecting clinical assessment of insulin sensitivity in horses, Equine Vet J, 2007. **39**(6): p. 567-575.

32. Firshman, A. M., S. J. Valberg, et al., Serum creatine kinase response to exercise during dexamethasone-induced insulin resistance in Quarter Horses with polysaccharide storage myopathy, Am J Vet Res, 2005. **66**(10): p. 1718-1723.

33. Forhead, A. J., J. C. Ousey, et al., Postnatal insulin secretion and sensitivity after manipulation of fetal growth by embryo transfer in the horse, J Endocrinol, 2004. **181**(3): p. 459-467.

34. Fowden, A.L., R.S. Comline, et al., Insulin secretion and carbohydrate metabolism during pregnancy in the mare, Equine Vet J, 1984. **16**(4): p. 239-246.

35. Frank, N., R. J. Geor, et al., Equine metabolic syndrome, J Vet Intern Med, 2010. **24**(3): p. 467-475.

36. Gannon, M.C., F.Q. Nuttall, et al., An increase in dietary protein improves to blood glucose response in persons with type 2 diabetes, Am J Clin Nutr, 2003. **78**(4): p.734-741.

37. Garcia, M.C. and J. Beech, Equine intravenous glucose tolerance test: glucose and insulin responses of healthy horses fed grain or hay and of horses with pituitary adenoma, Am J Vet Res, 1986. **47**(3): p. 570-572.

38. Geor, R. and N. Frank, Metabolic syndrome-From human organ disease to laminar failure in equids, Vet Immunol Immunopathol, 2009. **129**(3-4): p.151-154.

39. Geor, R. J. and P. Harris, Dietary management of obesity and insulin resistance: countering risk for laminitis, Vet Clin North Am Equine Pract, 2009. **25**(1): p. 51-65.

40. George, L. A., W. B. Staniar, et al., Evaluation of the effects of pregnancy on insulin sensitivity, insulin secretion, and glucose dynamics in Thoroughbred mares, Am J Vet Res, 2011. **72**(5): p. 666-674.

41. Goedecke, J.H., N.S. Levitt, et al., Differential effects of abdominal adipose tissue distribution on insulin sensitivity in black and white South African women, Obesity (Silver Spring), 2009. **17**(8): p. 1506-1512.

42. Graham, B. P., Dental care in the older horse, Vet Clin North Am Equine Pract, 2002. **18**(3): p. 509-522.

43. Gumbiner, B., K. S. Polonsky, et al., Effects of aging on insulin secretion, Diabetes, 1989. **38**(12): p. 1549-1556.

44. Gungor, N., R. Saad, et al., Validation of surrogate estimates of insulin sensitivity and insulin secretion in children and adolescents, J Pediatr, 2004. **144**(1): p. 47-55.

45. Harris, P., S. R. Bailey, et al., Countermeasures for pasture-associated laminitis in ponies and horses, J Nutr, 2006. **136**(7 Suppl): p. 2114S-2121S.

46. Harris, P. and R. J. Geor, Primer on dietary carbohydrates and utility of the glycemic index in equine nutrition, Vet Clin North Am Equine Pract, 2009. **25**(1): p. 23-27.

47. Henneke, D. R., G. D. Potter, et al., Relationship between condition score, physical measurements and body fat percentage in mares, Equine Vet J, 1983. **15**(4): p. 371-372.

48. Henriksen, E.J., Invited review:Effects of acute exercise and exercise training on insulin resistance, J Appl Physiol, 2002. **93**(2): p. 788-796.

49. Hoffman, R. M., R. C. Boston, et al., Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings, J Anim Sci, 2003. **81**(9): p. 2333-2342.

50. Hoffman, R. M., D. S. Kronfeld, et al., Glucose clearance in grazing mares is affected by diet, pregnancy, and lactation, J Anim Sci, 2003. **81**(7): p.1764-1771.

51. Hoffman, R. M., L. A. Lawrence, et al., Dietary carbohydrates and fat influence radiographic bone mineral content of growing foals, J Anim Sci, 1999. **77**(12): p. 3330-3338.

52. Holdstock, N. B., V. L. Allen, et al., Development of insulin and proinsulin secretion in newborn pony foals, J Endocrinol, 2004. **181**(3): p. 469-476.

53. Jackson, R.A., Mechanisms of age-related glucose intolerance, Diabet Care, 1990. **13**(Supplement 2): p. 9–19.

54. Jacobs, K.A. and J.R. Bolton, Effect of diet on the oral glucose tolerance test in the horse, J Am Vet Med Assoc, 1982. **180**(8): p. 884-886.

55. Jeffcott, L. B., J. R. Field, et al., Glucose tolerance and insulin sensitivity in ponies and Standardbred horses, Equine Vet J, 1986. **18**(2): p. 97-101.

56. Johnson, P. J., The equine metabolic syndrome peripheral Cushing's syndrome,Vet Clin North Am Equine Pract, 2002. **18**(2): p. 271-293.

57. Johnson, P. J., C. E. Wiedmeyer, et al., Laminitis and the equine metabolic syndrome, Vet Clin North Am Equine Pract, 2010. **26**(2): p. 239-255.

58. June, V., V. Soderholm, et al., Glucose tolerance in the horse, pony and donkey, Equine Vet Sci, 1992. **12**(2): p.103-105.

59. Kim, J.Y., L.A. Nolte, et al., High-fat diet-induced muscle insulin resistance: relationship to visceral fat mass, Am J Physiol Regul Integr Comp Physiol, 2000. **279**(6): p. R2057-2065.

60. Krezowski, P. A., F.Q Nuttall, et al., Insulin and glucose responses to various startch-containing foods in type II diabetic subjects, Diabetes Care, 1987, **10**(2): p. 205-212.

61. Kronfeld, D. S., Dietary fat affects heat production and other variables of equine performance, under hot and humid conditions, Equine Vet J Suppl 1996. (22): p. 24-34.

62. Kronfeld, D. S., K. H. Treiber, et al., Comparison of nonspecific indications and quantitative methods for the assessment of insulin resistance in horses and ponies, J Am Vet Med Assoc, 2005. **226**(5): p. 712-719.

63. Kronfeld, D. S., K. H. Treiber, et al., Metabolic syndrome in healthy ponies facilitates nutritional countermeasures against pasture laminitis, J Nutr, 2006. **136**(7 Suppl): p. 2090S-2093S.

64. Liburt, N.R., Fugaro, M.N., et al., The effect of age and exercise training on insulin sensitivity, fat and muscle tissue cytokine profiles and body composition of old and young Standardbred mares, Comp Exer Phys, 2012. **8**(3-4): p. 173-187.

65. Lowder, M. Q. and P. O. Mueller, Dental disease in geriatric horses, Vet Clin North Am Equine Pract, 1998. **14**(2): p. 365-380.

66. Lyon, C. J., R. E. Law, et al., Mini review: adiposity, inflammation, and atherogenesis." Endocrinology, 2003. **144**(6): p. 2195-2200.

67. Madigan, J. E. and J. W. Evans, Insulin turnover and irreversible loss rate in horses, J Anim Sci, 1973. **36**(4): p. 730-733.

68. Mair, T. S., M. H. Hillyer, et al., Small intestinal malabsorption in the horse: an assessment of the specificity of the oral glucose tolerance test, Equine Vet J,1991. **23**(5): p. 344-346.

69. Malinowski, K., C. L. Betros, et al., Effect of training on age-related changes in plasma insulin and glucose, Equine Vet J Suppl, 2002. (34): p. 147-153.

70. Maresh, M., Diabetes in pregnancy, Curr Opin Obstet Gynecol, 2001. **13**(2): p.103-107.

71. Masuzaki, H., J. Paterson, et al., A transgenic model of visceral obesity and the metabolic syndrome, Science, 2001. **294**(5549): p. 2166-2170.

72. McCue, M.E., R. J. Geor, et al., Equine metabolic syndrome: a complex disease influenced by genetics and the environment, Journal of Equine Veterinary Science, 2015, **35**(5): p. 367-375.

73. McEvoy, C.T., C.R. Cardwell, et al., A posteriori dietary patterns are related to risk of type 2 diabetes: findings from a systematic review and meta-analysis, J of Academy of Nutr and Dietetics, 2014. **114**(11): p. 1759-1775.

74. Menzies-Gow, N., Diabetes in the horse: a condition of increasing clinical awareness for differential diagnosis and interpretation of tests, Equine Vet J, 2009. **41**(9): p. 841-843.

75. Menzies-Gow, N. J., L. M. Katz, et al., Epidemiological study of pasture-associated laminitis and concurrent risk factors in the South of England, Vet Rec, 2010. **167**(18): p. 690-694.

76. Miller, M. A., I. D. Pardo, et al., Correlation of pituitary histomorphometry with adrenocorticotrophic hormone response to domperidone administration in the diagnosis of equine pituitary pars intermedia dysfunction, Vet Pathol, 2008. **45**(1): p. 26-38.

77. Milinovich, G. J., D. J. Trott, et al., Changes in equine hindgut bacterial populations during oligofructose-induced laminitis, Environ Microbiol, 2006. **8**(5): p. 885-898.

78. Morley, J. E., The aging gut: physiology, Clin Geriatr Med, 2007. **23**(4): p. 757-767, v-vi.

79. Morino, K., K. F. Petersen, et al., Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction, Diabetes, 2006. **55 Suppl 2**: p. S9-S15.

80. Murphy, D. Reid, S.W.J., et al., The effect of age and diet on the oral glucose tolerance test in ponies, Equine Vet J, 1997. **29**(6): p. 467-470.

81. Nauck, M., F. Stockmann, et al., Reduced incretin effect in type 2 (non-insulin-dependent) diabetes, Diabetologia,1986. **29**(1): 46-52.

82. Nakae, Y., H. Onouchi, et al., Effects of aging and gastric lipolysis on gastric emptying of lipid in liquid meal, J Gastroenterol, 1999. **34**(4): p. 445-449.

83. Nielsen, B.D., O'Connor-Robison, C.I., et al., Glycemic and insulinemic responses are affected by age of horse and method of feed processing, Journal of Equine Veterinary Science, 2010. **30**(10): p. 249-258.

84. Pagan, J. D., R. J. Geor, et al., Effects of fat adaptation on glucose kinetics and substrate oxidation during low-intensity exercise, Equine Vet J Suppl, 2002. (34): p. 33-38.

85. Paneni, F., S. Costantino, et al., Insulin resistance, diabetes, and cardiovascular risk, Curr Atheroscler Rep, 2014. **16**(7): 419.

86. Parillo, M., A.A. Rivellese, et al., A high-monounsaturated-fat/low-carbohydrate diet improves peripheral insulin sensitivity in non-insulin-dependent diabetic patients, Metabolism, 1992. **41**(12): p. 1373-1378.

87. Pawlak, D. B., J. A. Kushner, et al., Effects of dietary glycaemic index on adiposity, glucose homoeostasis, and plasma lipids in animals, Lancet, 2004. **364**(9436): p. 778-785.

88. Potau, N., L. Ibanez, et al., Pubertal changes in insulin secretion and peripheral insulin sensitivity, Horm Res, 1997. **48**(5): p. 219-226.

89. Powell, D.M., S.E. Reedy, et al., Effect of short-term exercise training on insulin sensitivity in obese and lean mares, Equine Vet J Suppl, 2002. (34): p. 81-84.

90. Pratt, S. E., R. J. Geor, et al., Effects of dietary energy source and physical conditioning on insulin sensitivity and glucose tolerance in standardbred horses, Equine Vet J Suppl, 2006.(36): p. 579-584.

91. Ralston, S. L., Insulin and glucose regulation, Vet Clin North Am Equine Pract, 2002. **18**(2): p. 295-304, vii.

92. Ralston, S. L., Evidence-based equine nutrition, Vet Clin North Am Equine Pract, 2007. **23**(2): p. 365-384.

93. Razzouk, L. and P. Muntner, Ethnic, gender, and age-related differences in patients with the metabolic syndrome, Curr Hypertens Rep, 2009. **11**(2): p. 127-132.

94. Roberts, M.C. and F.W. Hill, The oral glucose tolerance test in the horse, Equine Vet J, 1973. **5**(4): p. 171-173.

95. Roth, J., X. Qiang, et al., The obesity pandemic: where have we been and where are we going?, Obes Res, 2004. **12 Suppl 2**: p. 88S-101S.

96. Salles, N., Basic mechanisms of the aging gastrointestinal tract, Dig Dis, 2007. **25**(2): p. 112-117.

97. Schmidt, O., E. Deegen, et al., Effects of fat feeding and energy level on plasma metabolites and hormones in Shetland ponies, J Vet med A Physiol Pathol Clin Med, 2001. **48**(1): p. 39-49.

98. Schott, H.C., Pituitary pars intermedia dysfunction: equine Cushing's disease, Vet Clin North Am Equine Pract, 2002. **18**(2): p. 237-270.

99. Schuver, A., Frank, N., et al., Assessment of insulin and glucose dynamics by using an oral sugar test in horses, Journal of Equine Veterinary Science, 2014. **34**(4): p. 465-470.

100. Shen, S. W., G. M. Reaven, et al., Comparison of impedance to insulin-mediated glucose uptake in normal subjects and in subjects with latent diabetes, J Clin Invest, 1970. **49**(12): p. 2151-2160.

101. Shepherd, P. R. and B. B. Kahn, Glucose transporters and insulin action—implications for insulin resistance and diabetes mellitus, N Engl J Med, 1999. **341**(4): p. 248-257.

102. Shirazi-Beechey, S.P., Moran, A.W., et al., Influences of food constituents on gut health; glucose sensing and signaling; regulation of intestinal glucose transport, Proceedings of the Nutrition Society, 2011.**70**: p. 185–193.

103. Smyth, G. B., D. W. Young, et al., Maturation of insulin and glucose responses to normal feeding in foals, Aust Vet J, 1997. **70**(4): p. 129-132.

104. Stuart, C. A., M.E. Howell, et al., Insulin-stimulated translocation of glucose transporter (GLUT) 12 parallels that of GLUT4 in normal muscle, J Clin Endocrinol Metab, 2009. **94**(9): p. 3535-3542.

105. Suagee, J.K., B.A. Corl, et al., Effects of hyperinsulinemia on glucose and lipid transporter expression in insulin-sensitive horses, Domest Anim Endocrinol, 2011. **40(**3): p. 173-181.

106. Suagee, J.K., B.A. Corl, et al., A 90-day adaptation to a high glycaemic diet alters postprandial lipid metabolism in non-obese horses without affecting peripheral insulin sensitivity, J Anim Physiol Anim Nutr (Berl), 2013. **97**(2): p. 245-254.

107. Suagee, J. K., B. A. Corl, et al., Relationships between body condition score and plasma inflammatory cytokines, insulin, and lipids in a mixed population of light-breed horses, J Intern Med 2013. **27**(1): p. 157-163.

108. Tinworth, K.D., P.C. Wynn, et al., Evaluation of commercially available assays for the measurement of equine insulin, Domest Anim Endocrinol, 2011. **41**(2): p. 81-90.

109. Toth, F., N. Frank, et al., Optimization of the frequently sampled intravenous glucose tolerance test to reduce urinary glucose spilling in horses, Equine Vet J, 2009. **41**(9): p. 844-851.

110. Treiber, K. H., R. C. Boston, et al., Insulin resistance and compensation in Thoroughbred weanlings adapted to high-glycemic meals, J Anim Sci, 2005b. **83**(10): p. 2357-2364.

111. Treiber, K. H., T. M. Hess, et al., Glucose dynamics during exercise: dietary energy sources affect minimal model parameters in trained Arabian geldings during endurance exercise, Equine Vet J Suppl, 2006. (36): p. 631-636.

112. Treiber, K. H., D. S. Kronfeld, et al., Use of proxies and reference quintiles obtained from minimal model analysis for determination of insulin sensitivity and pancreatic beta-cell responsiveness in horses, Am J Vet Res, 2005. **66**(12): p. 2114-2121.

113. Treiber, K. H., D. S. Kronfeld, et al., Evaluation of genetic and metabolic predispositions and nutritional risk factors for pasture-associated laminitis in ponies, J Am Vet Med Assoc, 2006a. **228**(10): p. 1538-1545.

114. Treiber, K. H., D. S. Kronfeld, et al., Insulin resistance in equids: possible role in laminitis, J Nutr, 2006b. **136**(7 Suppl): p. 2094S-2098S.

115. Tschritter, O., A. Fritsche, et al., Assessing the shape of the glucose curve during an oral glucose tolerance test, Diabetes Care, 2003. **26**(4): p. 1026-1033.

116. Vervuert, I, S. Klein, et al., Effects of feeding state on glycaemic and insulinaemic responses to a starchy meal in horses: a methodological approach, Animal, 2009, **3**(9): p. 1246-1253.

117. Vick, M. M., A. A. Adams, et al., Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse, J Anim Sci, 2007. **85**(5): 1144-1155.

118. Volek, J.S. and R.D. Feinman, Carbohydrate restriction improves the features of Metabolic Syndrome. Metabolic Syndrome may be defined by the response to carbohydrate restriction, Nutr Metab (Lond), 2005. **2**: p. 31.

119. Waller, A. P., T. A. Burns, et al., Insulin resistance selectively alters cell-surface glucose transporters but not their total protein expression in equine skeletal muscle, J Vet Intern Med, 2011. **25**(2): 315-321.

120. Waller, A.P., L. Huettner, et al., Novel link between inflammation and impaired glucose transport during equine insulin resistance, Vet Immunol Immunopathol, 2012. **149**(3-4): P.208-215.

121. Waller, A.P., M. George, et al., GLUT 12 functions as a basal and insulin-independent glucose transporter in the heart, Biochem Biophys Acta, 2013. **1832**(1): p. 121-127.

122. Warren, L.K., Vineyard, K.R., Fat and fatty acids. In: Equine Applied and Clinical Nutrition. Elsevier, St. Louis, MO, USA, 2013: p. 136-155.

123. Watson, R.T., Pessin, J.E., Intracellular organization of insulin signaling and GLUT4 translocation. Recent Progress in Hormone Research, 2011. (56): p. 175-193.

124. Watts, K., Pasture management to minimize the risk of equine laminitis, Vet Clin North Am Equine Pract, 2010. **26**(2): p. 361-369.

125. Williams, C.A., D.S. Kronfeld, et al., Plasma glucose and insulin responses of Thoroughbred mares fed a meal high in starch and sugar or fat and fiber, J Anim Sci, 2001. **79**(8): p. 2196-2201.

126. Xiao, Z. Q., L. Moragoda, et al., Aging is associated with increased proliferation and decreased apoptosis in the colonic mucosa, Mech Ageing Dev, 2001. **122**(15): p. 1849-1864.

127. Xu, H., G. T. Barnes, et al., Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance, J Clin Invest, 2003. **112**(12): p. 1821-1830.